

University of Nevada, Reno

Immunology and Disease in the Mojave Desert Tortoise (*Gopherus agassizii*)

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Ecology, Evolution, and Conservation Biology

by

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prepared under our supervision by

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ABSTRACT

The motivation for this study was to gain a better understanding of the possible effects of upper respiratory tract disease (URTD) and *Mycoplasma agassizii* (an etiological agent of URTD) in the Mojave desert tortoise, a federally-threatened population. We hope to influence future ecological studies as well as conservation strategies in the management of wild populations of the desert tortoise. Specifically, we (1) reviewed the entire published literature of URTD in Mojave desert tortoises, (2) measured aspects of a generalized acquired immune response in desert tortoises in a controlled environment, and (3) conducted a range-wide survey of URTD and the seroprevalence of *M. agassizii* in Mojave desert tortoises. (1) In the review of the literature we challenge the view that *M. agassizii* causes consistent levels of morbidity and/or mortality across the Mojave Desert. Instead, URTD may be described more accurately as a context-dependent disease. We summarize new evidence of relatively high levels of natural antibodies to *M. agassizii* in desert tortoises, which suggests possible problems of conventional diagnostic tests and a possible tortoise immune mechanism of defense against *M. agassizii*. Partly because of the problems with diagnostic testing, we recommend abandoning policies to euthanize tortoises that test positive for an immune response to *M. agassizii*. Based on this review, we question management strategies aimed solely at reducing *Mycoplasma spp* in desert tortoise populations, and advocate a more careful consideration of extrinsic factors as an additional, potential cause of disease. (2) We induced an acquired, humoral (antibody) response in desert tortoises, via immunization with ovalbumin (OVA). We immunized

tortoises both before and after hibernation, and observed a gender-by-season interaction in the ability of desert tortoises to make an induced immune response. We observed relatively high levels of pre-existing natural antibody to OVA in all tortoises, and levels varied among individuals. There was a significant, negative relationship between an animal's natural antibody level and the maximum increase in acquired antibody levels. There was a significant, positive relationship between the magnitude of long-term elevations in OVA-specific antibody levels and maximum increase in acquired levels. This experiment suggested that both natural and long-term elevations in acquired antibody levels may be important elements of the tortoise immune system, with possible influences on the ecology and evolution of host-pathogen interactions. (3) We focused our range-wide survey on population-level analyses ($n = 24$), and tested for associations among the prevalence of URTD, seroprevalence to *M. agassizii*, genetics of tortoise populations, mean annual winter precipitation, and mean number of days below freezing. We detected significant associations between mean number of days below freezing and both the prevalence of URTD and the seroprevalence to *M. agassizii*. Furthermore, we detected a significant association between mean levels of natural antibody and seroprevalence to *M. agassizii*. Genetics of tortoise populations was associated with mean levels of natural antibody. We propose hypotheses, concerning possible ecological and evolutionary dynamics of the desert tortoise – *M. agassizii* system, based on these associations and specific recommendations for future research to test these hypotheses.

DEDICATION

To two desert tortoises, Poncho and L16, who were euthanized during the course of basic research, which contributed to the immunological foundations of this study. Poncho was a tortoise collected in Clark County, and deemed as “URTD-positive” and therefore slated for euthanasia. L16 had been part of a previous experiment at another research institution (UNLV), before he was moved to UNR. Their tissues have led to a better, basic understanding of aspects of the immune system of desert tortoises. Their deaths have helped save the lives of other tortoises in Nevada.

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Chapter 1. Upper Respiratory Tract Disease (URTD) as a Threat to Desert Tortoise Populations: A Reevaluation

Abstract

The relationships between *Mycoplasma agassizii*, a causative agent of upper respiratory disease (URTD), and desert tortoise (*Gopherus agassizii*), generally illustrate the complexities of disease dynamics in wild vertebrate populations. In this review, we summarize current understanding of URTD in Mojave desert tortoise populations, we illustrate how inadequate knowledge of tortoise immune systems may obfuscate assessment of disease, and we suggest approaches to future management of URTD in desert tortoise populations. We challenge the view that *M. agassizii* causes consistent levels of morbidity and/or mortality across the Mojave Desert. Instead, URTD may be described more accurately as a context-dependent disease. In addition, new evidence for relatively high levels of natural antibodies to *M. agassizii* in desert tortoises suggests possible problems in conventional diagnostic tests of disease in tortoises as well as a possible tortoise immune mechanism to protect against *M. agassizii*. Partly because of the problems in diagnostic testing, we recommend abandoning policies to euthanize tortoises that test positive for an immune response to *M. agassizii*. Based on this review, we question management strategies aimed solely at reducing *Mycoplasma spp* in desert tortoise populations, and advocate a more careful consideration of extrinsic factors as a cause of symptomatic disease.

Keywords

natural antibody, wildlife disease, opportunistic, epidemiology, desert tortoise, conservation

Introduction

The Mojave population of the desert tortoise, *Gopherus agassizii*, was listed as threatened under the U.S. Endangered Species Act in 1990, in part due to observations of “upper respiratory tract disease” (URTD) in wild populations (FWS, 1990, 1994). URTD is a description of symptoms that include nasal exudate, edema around the eyes, histological lesions in the nasal epithelium and in the mucosa of the upper respiratory tract, and in severe cases, lethargy and death (Brown et al., 1994; Berry and Christopher, 2001). Although *Mycoplasma agassizii* has been experimentally shown to be one causative agent (Brown et al., 1994) (see Table 1), URTD in the desert tortoise and gopher tortoise (*Gopherus polyphemus*) has also been associated with other pathogens, such as *Pasteurella testudinis* (desert tortoise: Snipes and Fowler, 1980; Jacobson et al., 1991; Snipes et al., 1995; Dickenson et al., 2001), an iridiovirus (gopher tortoise: Westhouse et al., 1996), and herpes virus infections (Pettan-Brewer et al., 1996; Johnson et al., 2005; Jacobson, 2007).

URTD has been considered an important threat to persistence of desert tortoise populations and as a threat that should be mitigated as part of the recovery of the species (FWS, 1994; Tracy et al., 2004). The 1989 emergency listing of the Mojave population as endangered was, in part, justified by initial observations of URTD in the Desert Tortoise Natural Area (DTNA) in the western Mojave of California, and by the interpretation that

this disease was a possible novel epidemic with the potential to spread across desert tortoise populations of the Mojave desert (FWS, 1989). However, one reason for the subsequent down-listing of the Mojave desert tortoise from endangered to threatened status stemmed from the recognition that the severity of URTD did not seem to be similarly severe across the range for the Mojave desert tortoise (FWS, 1990). Roughly concurrent with the listing of the Mojave desert tortoises as threatened in 1990, URTD was implicated in population declines (Berry, 1990; FWS, 1990, 1994). In particular, documented mortality among desert tortoise appeared to be severe at the Desert Tortoise Natural Area in the Western Mojave of California, which correlated with concurrent high incidences of symptoms of URTD in 1988-1990 (Table 2) (e.g. Berry, 1990). However, plot-based surveys suggest that mortality was more severe at the DTNA plot than in other plots in the Mojave (Corn, 1994). Although declines in some areas of the Western Mojave have been corroborated by additional data (Corn, 1994; Tracy et al., 2004), there are inadequate data documenting abnormal range-wide population declines (Corn, 1994; Germano and Bury, 1994; Bury and Corn, 1995; Tracy et al. 2004)

Management of URTD

Despite recognized uncertainties in the extent and severity of URTD in natural tortoise populations across the Mojave, URTD certainly could pose a serious threat to desert tortoise populations, and conservation prescriptions have taken the spread of URTD into consideration when management actions have involved handling and/or moving wild tortoises (FWS, 1994; Tracy et al., 2004). In particular, urban development projects have sometimes necessitated the translocation of wild animals into protected or undeveloped areas. For example, as of 2006, the rapid expansion of Las Vegas and other

cities in Clark County, Nevada, had displaced 16,507 tortoises that have been collected and moved to a temporary holding facility (Tracy, unpublished data). At the holding facility, the tortoises are tested for exposure to *M. agassizii* by an enzyme-linked immunosorbent assay (ELISA). That test detects levels of *M. agassizii*-specific antibodies in tortoise blood serum. Tortoises that test sero-negative are typically translocated to a “large scale translocation site” southwest of Las Vegas. Animals that are classified as URTD “suspect” or “positive” by the ELISA have been euthanized. Euthanasia has been considered as a conservative approach to protect potentially healthy wild desert tortoise populations (Berry and Slone, 1989; Jacobson et al. 1995). Of the 16,507 tortoises that have been collected in Clark County since 1990, 3,237 have been programmatically euthanized (Tracy, unpublished data). Euthanasia was management policy from 1990-2006.

Largely due to the recent recognition of uncertainty in the ability to diagnose URTD in tortoises, apparent populational differences in manifestation of URTD across the Mojave, and a realization that a disturbingly large number of tortoises have been euthanized, tortoises labeled as being “suspect” and “positive” with respect to URTD are currently no longer euthanized. Instead, they are maintained in separate pens at the Desert Tortoise Conservation Center, Clark Co., NV. These tortoises will contribute to research projects designed to further our understanding of this disease and its transmission (Draft Recovery Plan for the Mojave Desert Tortoise, Nevada Field Office, FWS, 2007).

Despite an increasing interest in the epidemiology of wildlife diseases in conservation biology (Cleaveland et al., 2002), there are apparent gaps between the methods and research designs of immunologists and those of ecologists (Norris and

Evans, 2000; Salvante, 2006). In addition, the management of disease in non-mammalian vertebrates, such as ectotherms, is complicated by the relative dearth of knowledge about ectotherm immune systems (Manning, 1994; Horton, 1994; Jurd, 1994). The desert tortoise-*M. agassizii* system is an important case study in what must be accomplished in order to respond to mandates from conservation policy vis-a-vis wildlife disease. For the desert tortoise, there is a pressing need to conduct basic immunological research on the host species as well as a need to learn more about the epidemiology of pathogens across the natural extent of host populations.

Objectives of this review

We review hypotheses about the desert tortoise-*M. agassizii* interactions and existing diagnostic techniques to assess the presence of *M. agassizii* in individual tortoises. This review has been influenced by recent immunological research suggesting that desert tortoises produce natural antibodies to *M. agassizii*, which are considered to be a component of the tortoise innate immune system (Hunter et al., 2008). High levels of *M. agassizii*-specific natural antibodies suggest the possibility of a long evolutionary relationship between desert tortoises and *Mycoplasma* spp. These antibodies also suggest a biological reason for previously noted high “background” antibody levels in ELISA tests, which can be a source of inaccuracy in current *Mycoplasma*-specific diagnostic tests (Hunter et al., 2008; Schumacher et al., 1993).

We focus largely on roughly 18 years of research on URTD, including all peer-reviewed publications, as well as influential “gray literature” cited in the Desert Tortoise Recovery Plan (FWS, 1994). A re-interpretation of the literature shows that an accumulation of relatively small-scale field studies has increased our understanding of

this host-pathogen system over space and time. In addition, new immunological research is increasing our understanding of the mechanisms underpinning the desert tortoise immune response. We present an evaluation of the literature and new immunological knowledge to: (1) critically summarize this host-pathogen system and the efficacy of the current disease diagnosis via ELISA, (2) challenge the hypothesis that URTD is an epidemic phenomenon that has the intrinsic ability to cause widespread population declines in desert tortoises, (3) discuss the implications of new evidence indicating that natural antibodies in the desert tortoise account for apparent high “background” levels of *M. agassizii*-specific antibodies, and (4) assess the appropriateness of management practices involving euthanasia of ELISA-positive animals. More generally, we aim to show the value to conservation biology in more fully understanding host-pathogen ecological systems, including mechanisms of host immunology and the importance of context-dependency of some wildlife diseases.

Host-pathogen system and disease diagnosis by ELISA testing

Mycoplasmosis in desert tortoises

Five species of *Mycoplasma*, belonging to the bacterial class of Mollicutes (Barile et al., 1985) have been identified in the desert tortoise. Three of these species have been named (Brown et al., 1995, 2001, 2002). *M. agassizii* and *M. testudineum* both infect the respiratory tract and can cause symptoms of URTD in desert and gopher tortoises (Brown et al., 1994, 1999a, 2004). The third species, *M. testudinis*, has only been isolated from the cloaca of desert tortoise, and it is not thought to cause symptoms of URTD (Brown et al., 1995, 2002). *M. testudineum* has only recently been recognized as a distinct species, but because of its close relationship to *M. agassizii* (Brown et al., 2004), it is assumed

that some of the diagnostic techniques used to detect *M. agassizii* in the desert tortoise should also detect *M. testudineum*. For clarity, we will refer to *M. agassizii* explicitly when citing those studies that only focused on this species. “*Mycoplasma* spp” will be used to include all species of *Mycoplasma* that may be involved in respiratory mycoplasmosis in desert tortoises.

Species of mycoplasmas have been discovered in a diversity of vertebrate hosts (Simecka et al., 1992; Stipkovits and Kempf, 1996; Razin et al., 1998; Brown, 2002; Rottem, 2003). Many of these pathogens are opportunistic, causing clinical symptoms of disease only in conjunction with extrinsic factors, such as compromised host immunocompetence or concomitant infection with other pathogens (Simecka et al., 1992; Stipkovits and Kempf, 1996; Razin et al., 1998; Cassell et al., 1985; Waites and Talkington, 2004). However, the focus of most research in desert tortoise populations has been on the presence of antibodies to *M. agassizii*, and no studies have tried to quantify the potential interaction of infection with *Mycoplasma* spp with extrinsic factors that may alter pathogen prevalence or virulence or host susceptibility to infection and disease. In addition, no study has examined whether high levels of *Mycoplasma* – specific antibodies in wild tortoises actually protect against developing severe URTD. Such a relationship might indicate that the most “sero-positive” individuals actually are the most resistant to URTD. Many studies have suggested that the presence of *M. agassizii* is sufficient to explain observed morbidity and mortality associated with symptoms of URTD in desert tortoise populations (Jacobson et al., 1991, 1995; Schumacher et al., 1993, 1997; Brown et al., 1994, 1999b; FWS, 1994; Christopher et al., 2003), but this hypothesis largely remains untested.

Definition of “mycoplasmosis”

In the tortoise literature, the terms “URTD”, disease, “mycoplasmosis”, and infection often have not been clearly defined. This may be due to the current technical inability to differentiate between all possible health states, which in the medical literature are often referred to as “naïve”, “colonized”, “infected”, and “diseased” (sensu American Heritage Medical Dictionary, 2007; Blood et al., 2007). Within this framework of terminology, a naïve animal is unexposed to a particular microorganism. A colonized animal’s tissues have adherent pathogens, or pathogens that have breached the epithelial barrier without detectable local or systemic damage. Colonization can also be described as a “latent infection”, or the persistence of the pathogen in the host without sufficient replication and pathology to cause disease. An infected animal’s tissues have been invaded, and the pathogen has caused either a local or systemic physiological response. A diseased animal is an infected animal with observable symptomatic disease. Although colonization may or may not produce a detectable adaptive immune response, infection usually induces a clear adaptive immune response in vertebrates, except in severely immunocompromised individuals. These are general terms used most often in the human medical literature.

An “infection” is used slightly differently in epidemiological models within the ecological literature. In the ecological literature, “infected” individuals include all those that may transmit the disease to others, regardless of whether they are colonized or infected and regardless of whether they manifest overt versus subclinical symptoms of disease. Most diseases that are commonly modeled are highly infectious and pathogenic, and in those cases a clear distinction between colonized and infected individuals may be

unnecessary. When this distinction does appear to affect disease dynamics, such models may simply include a “lag time” to account for the time it takes for an exposed individual to become infectious to others.

Differences in definitions of health status become important when diseases involve opportunistic pathogens. Opportunistic pathogens are organisms that do not ordinarily cause disease, but become pathogenic under certain circumstances, such as when host immune function is impaired (e.g. Blood et al., 2007). Two of the most commonly recognized causes of impaired immune function include inadequate nutrition and concurrent infections with other pathogens (e.g. Wobeser, 2006). Therefore, opportunistic pathogens may colonize hosts under certain conditions without causing harm, yet they may cause harmful infectious disease under other conditions.

Measurements of the presence of *M. agassizii* in desert tortoises have relied largely on indirect measurements, such as tortoise antibody production. However, relationships among adaptive antibody production, the local abundance of *M. agassizii* present within an individual host, and the degree of local or systemic damage caused by *M. agassizii* remains unknown. Clear distinctions between naïve, colonized, and infected desert tortoises are currently not possible.

Within this review, tortoises are described as “URTD-positive”, or “diseased” if they show symptoms of URTD regardless of the cause. “Mycoplasmosis” as used refers to tortoises mounting an induced, or adaptive, antibody response to *M. agassizii* and/or related *Mycoplasma* spp. Although the term “mycoplasmosis” implies infection, it is important to recognize that these tortoises have simply been exposed to *Mycoplasma* spp. Without reliable diagnostic tests to measure *Mycoplasma* spp directly, we are currently

neither able to assess the presence and amount of mycoplasma, nor are we able to estimate the degree of local or systemic harm associated with the presence of mycoplasma in the respiratory tract of live desert tortoises.

In summary, as used in this review, a “mycoplasmosis-positive” tortoise has a detectable current or recent colonization/infection with *Mycoplasma* spp (via an induced antibody response), regardless of the degree of local tissue harm (e.g. lesions in respiratory tract epithelia) or symptomatic URTD. Indeed, a tortoise diagnosed as mycoplasmosis-positive also could be an animal that has recovered from an infection, and which has cleared the pathogen from its body. On the other hand, if a tortoise is not making an induced antibody response, several possibilities could be true. The tortoise may be truly uninfected, never having been exposed to *Mycoplasma* spp. The tortoise may be colonized by essentially “commensal” species or strains of *Mycoplasma* spp which are not causing tissue damage and are not inducing an adaptive immune response. The tortoise may be colonized (or infected) by *Mycoplasma* spp that are causing low levels of local tissue damage but again, not inducing an immune response. This would be an example of “subclinical” disease. Finally, the tortoise could be invaded with locally and/or systemically pathogenic *Mycoplasma* spp, but the tortoise is immunosuppressed and cannot make an antibody response. Due to the current lack of means to differentiate among these different states, we refer to any tortoise without an induced antibody response to *Mycoplasma* spp as “mycoplasmosis negative”.

Diagnosis of mycoplasmosis

A monoclonal ELISA test (Schumacher et al., 1993) has been used in both management and research, and it is considered the standard method for determining both

(a) an individual animal's exposure to *M. agassizii*, and (b) the seroprevalence in wild tortoise populations (Jacobson et al., 1991, 1995; Lederle et al., 1997; Schumacher et al., 1997; Brown et al., 1999b; Christopher et al., 2003; Dickinson et al., 2005). In addition to the ELISA, and the less stringent description of the clinical symptoms of URTD, there are several other procedures currently available to diagnose URTD. These include detecting the presence of *M. agassizii* DNA via the polymerase chain reaction (PCR) technique, or culture of microorganisms from nasal lavage samples (Brown et al., 2002). Both of these techniques are logistically difficult to perform accurately on samples collected from wild tortoises, and therefore are less sensitive than other diagnostic techniques (Brown et al., 2002). Although not currently available, improvements in the accurate direct detection of *M. agassizii* (e.g. via PCR or antigen assays) could be used to confirm or re-evaluate serological assays (e.g. ELISA and Western blot techniques), as well as to diagnose mycoplasmosis in individual tortoises. Histopathologic analysis of tissues from the respiratory tract of necropsied animals (which requires autopsy of a dead tortoise) has also been employed on a small scale (Brown et al., 2002; Wendland et al., 2007). The relatively minor invasiveness, low cost, and ease of processing large quantities of samples using ELISA have all led to a reliance on the monoclonal ELISA. For example, in Clark County, the need to manage large numbers of diseased, displaced tortoises has led to a sole reliance on ELISA results (Tracy, unpublished data).

ELISA

Due to its widespread application, it is important to recognize the inherent limitations of the currently used monoclonal ELISA (Brown et al., 2002). An ELISA test is an indirect measurement of pathogen exposure, and it only has the ability to detect the

current or past production of antibodies in the peripheral blood instead of assaying the actual presence and/or abundance of pathogen at a discrete point in time (Brown et al., 2002). Briefly, an ELISA measures serum or plasma antibody levels through a number of procedures that culminate in an enzymatic reaction. The enzymatic reaction is detected as a color change and measured as light absorbance in optical density units (OD). Results of blood serum samples tested via ELISA are usually reported as either end-point titer values (the serial dilution at which the OD approximates background absorbance) or as the absorbance at a single, selected dilution. The particular monoclonal ELISA currently in use for detecting mycoplasmosis measures absorbances at two dilutions to estimate an end point titer from calibration curves using full dilution curves taken to the end-point titer values (Brown et al., 2002). The ELISA used in the literature cited in this review had a reported sensitivity of >90%, a specificity of >85%, mean positive and mean negative predictive values of about 88%, and rates of false positives between 0-27% (Brown et al., 2002). The ELISA was recently refined and has a sensitivity of 98%, a specificity of 99%, and positive and negative predictive values of 90% or greater (when the population seroprevalence is between 9% and 85%) (Wendland et al., 2007). The ELISA is not commercially available, and samples are run at the University of Florida at Gainesville, FL (described in Wendland et al., 2007).

While a certain amount of subjectivity is inherent in any clinical assay, the greatest problem of this particular ELISA test is that no true “gold standard” was used to determine the test’s sensitivity and specificity, and to test its population-specific positive and negative predictive values (Brown et al., 2002; Loong, 2003). A “gold standard” is an independent standard used in determining whether an individual is truly positive or

negative, and can therefore be used in the validation of a separate assay (Loong, 2003). In human biomedicine, Western blots are routinely used as a confirmatory test to designate true positive and true negative subjects. Western blots can be used to verify that the antibody binding measured in the ELISA is specific to certain pathogen proteins, or antigens, resolved in the Western blot, and, for example, have been used in the validation of an ELISA to detect antibodies to HIV (Gürtler, 1996; Mas et al., 1997; Kleinman et al., 1998; Kassler et al., 1995; CDC, 1989, 1992). Although Schumacher et al. (1993) presented Western blots of three individual desert tortoises, Western blots were not subsequently used as an independent confirmatory test in ELISA validation. Schumacher et al. (1993) recognized the problem of not having true positive and negative control animals in their assessment of the ELISA, due to the current difficulties in accurate detection of *M. agassizii* by culture or PCR. They used pathologic and histologic evaluations of necropsy specimens to determine “true” health status (Schumacher et al., 1993). However, determinations of truly infected animals have not been consistent among subsequent studies. These subsequent studies approximated positive and negative predictive values of the ELISA by using different combinations of the presence of clinical signs and/or histopathological lesions (Brown et al., 1994, 1999b, 2002; Schumacher et al., 1997).

Given the great reliance on the current monoclonal ELISA, and somewhat incomplete knowledge of chelonian populations of antibody molecules (Benedict and Pollard, 1972; Coe, 1972; Ambrosius, 1976; Herbst and Klein, 1995; Turchin and Hsu, 1996), it is surprising that there has been no discussion in the desert tortoise literature of the different advantages of monoclonal ELISAs versus polyclonal ELISAs, both of which

have been used in studies in mammalian immunology (Janeway et al., 2005). In particular, there is no published comparison between these two types of ELISAs in measuring *M. agassizii*-specific tortoise antibodies (but see Schumacher and Klein, unpublished data, cited in Schumacher et al., 1993). The current monoclonal ELISA was created to recognize a light chain of desert tortoise antibody isotype IgY (Schumacher et al., 1993). This monoclonal antibody is reported to recognize all *M. agassizii*-specific IgM, IgY, and IgY(Δ)Fc in the desert tortoise, because these antibody molecules are expected to be made up of different heavy chains, but equivalent light chains (Schumacher et al., 1993; Brown et al., 2002). However, many vertebrates have more than one type of light chain, and the proportion of these light chains in antibodies varies greatly from species-to-species (Pilström et al., 1998). Research is needed to quantify the number, and relative proportion, of light chains in the desert tortoise to interpret data correctly that is obtained through the use of the monoclonal ELISA described in Schumacher et al. (1993). Polyclonal ELISAs recognize all heavy and light antibody chains and could be used to assess the monoclonal ELISA (Schumacher et al., 1993) to verify the assumption that the monoclonal ELISA is truly measuring all *M. agassizii*-specific tortoise antibodies (but see Schumacher and Klein, unpublished data, cited in Schumacher et al., 1993).

Current cutoff values of the *M. agassizii*-specific monoclonal ELISA reportedly were chosen conservatively for the purpose of minimizing false negative results (Brown et al., 2002; Wendland et al., 2007). Values reduced the chance of falsely declaring a tortoise free of mycoplasmosis and of translocating a potentially sick tortoise into a new tortoise population. However, this assay bias increases the chance of making false

positive errors, thereby increasing the chance of declaring an uninfected tortoise ill. In Clark County, NV, this bias increased the chance of euthanizing uninfected animals, and even a false positive error of only 10% could have resulted in more than 300 tortoises being euthanized even though they were healthy (Tracy, unpublished data).

During the ELISA's early use in the 1990's by researchers and managers, the ELISA assay was improved and the cut-off values used in differentiating between positive and negative animals were changed (Lederle et al., 1997). This serves as an example of the possible subjectivity in ELISA diagnoses. Lederle et al. (1997) calculated that this change in assay interpretation actually decreased the proportion of seropositive animals detected in their study from a putative 43% (using the "old" cut-off values) to only 19% (reported in their publication) (Lederle et al., 1997). Such levels of change in interpretation of ELISA results can affect the comparability of studies carried out before and after the change in cut-off values.

Alternative hypotheses

Hypotheses

A discussion of comprehensive, multiple alternative hypotheses concerning the biological significance of *Mycoplasma* spp in natural populations is missing in the desert tortoise literature. We propose possible alternative hypotheses, based on the wide range of effects that infective micro-parasites may have on host populations (sensu Wobeser, 2006). Hypothesis (1) is overly simplistic, because it has been assumed to be true in most of the literature on desert tortoise. We discuss the preponderance of Hypothesis (1) in the literature, as well as the inadequacy of data clearly supporting either Hypotheses (1) or

- (2). We provide evidence in support of Hypothesis (2), focusing on mechanisms (a) and (b). Essentially no information exists pertaining to mechanism (c).

Significance of Mycoplasma spp in tortoise populations (mutually exclusive alternatives)

(Hypothesis 1) *Mycoplasma* spp have the intrinsic ability to cause consistently high rates of morbidity and mortality in natural populations of the desert tortoise. As a correlate, we should be able to predict URTD-induced morbidity and mortality relatively accurately primarily by measuring the prevalence of *Mycoplasma* spp in tortoise populations.

(Hypothesis 2) *Mycoplasma* spp do not have the intrinsic ability to cause consistent rates of morbidity and mortality in natural desert tortoise populations. The relationship between the incidence of *Mycoplasma* spp and the incidence of clinical URTD should be temporally and/or spatially heterogenous. As a correlate, we should be able to predict URTD-induced morbidity and mortality only by measuring other factors in addition to the prevalence of *Mycoplasma* spp in tortoise populations.

Possible mechanisms for hypothesis 2 (not mutually exclusive alternatives)

- a) The ability of *Mycoplasma* spp to cause disease (pathogenicity) is influenced by extrinsic factors affecting the host immune response and/or pathogen abundance and virulence. These extrinsic factors may include drought, chronic stress, and other pathogens. Thus, *Mycoplasma* spp should be regarded as opportunistic pathogens.
- b) Different strains of *Mycoplasma* spp are not equally pathogenic, and genetic differences result in varying rates of morbidity and mortality in the host (desert tortoise).

- c) Different desert tortoise populations have different levels of genetic resistance to *Mycoplasma* spp.

Desert tortoise declines attributed to URTD [Hypothesis (1)]

Large declines in tortoise population density in the western Mojave were originally attributed, in part, to the occurrence of URTD (Berry, 1990, 1997), and these data have been widely referred to in the published literature (e.g. Brown et al., 1999a, 1999b; Berry and Christopher, 2001; Christopher et al., 2003), in the Federal Registers for the emergency listing of the tortoise in 1989 (FWS, 1989), in its subsequent listing as a threatened species (FWS, 1990), and in the Recovery Plan (FWS, 1994). Berry (1990) estimated tortoise density declines from seven sufficiently sampled plots in the western Mojave region over the course of approximately nine years (1979–1989) (Table 2). By plot, estimated tortoise density declines ranged from no significant decline to a 68% decline (Table 2). Incidence of clinical URTD ranged from 0% to 51% of surveyed tortoises showing some symptoms of URTD (Table 2). At the time of federal listing of the Mojave desert tortoise as threatened, observations of URTD-symptomatic animals warranted attention, but the prevalence of URTD was neither homogenous across the landscape, nor consistently linked to significant decreases in population density.

Caveats in historic data

Even with correlation between URTD and population declines, past studies did not control for common correlations with yet other variables such as site, year, climatic factors, trends in other potential stressors, changes in predominant forage species due to exotic invasions, or genetic traits of the host and pathogen populations.

For example, conditions caused by short-term drought have been shown to produce patterns of mortality similar to those that are expected to result from the occurrence of epidemic disease (Peterson, 1994; Longshore et al., 2003). A correlation between drought and high adult tortoise mortality has been observed in several studies of desert tortoise populations (Woodbury and Hardy, 1948; Peterson, 1994; reviewed in Longshore et al., 2003). Because three distinct causes of desert tortoise mortality – increased predation, starvation, and increased incidence of UR TD – are all correlated with periods of short-term drought (Peterson, 1994; Longshore et al., 2003), observations of UR TD and concurrent high mortality rates do not indicate a clear cause-and-effect relationship.

The study plots used to conduct historic tortoise surveys were relatively small (generally on the order of 2.6 km²) and did not cover an adequate extent of tortoise habitat to draw conclusions that may be extrapolated to entire Mojave desert tortoise populations (*sensu* Wiens, 1989; Corn, 1994). The original study plots also had been selected non-randomly, in favor of areas with particularly high tortoise densities (Corn, 1994). Such non-random research design may be particularly important when considering disease epidemiology, because the incidence of some diseases are known to increase with increasing host density (Anderson and May, 1991; Hudson et al., 2002; Wobeser, 2006). Thus, early observations of mortality and disease on desert tortoise study plots may not have been representative of more widespread desert tortoise population dynamics (Corn, 1994), nor of disease prevalence.

Despite a lack of data demonstrating predictably high levels of morbidity and/or mortality directly caused by *M. agassizii* over space and time, both peer-reviewed

(Schumacher et al., 1993; Brown et al., 1994, 1990b, 2001; Jacobson et al., 1995; Berry et al., 2002; Christopher et al., 2003) and influential non-peer-reviewed (e.g. Berry, 1990; Berry and Slone, 1989) literature assert a causal link between population declines and UR TD in natural populations. In addition, several publications refer to UR TD as an epidemic or epizootic, a claim that is similarly not based in evidence (Schumacher et al., 1993; Jacobson et al., 1995; Christopher et al., 2003). Many of these assertions seem to trace back to the initial observations of significant numbers of UR TD symptomatic animals and correlated declines in population density, predominantly in Fenner Valley and the DTNA in the western Mojave (Table 2) (e.g. Berry, 1990, 1997; Brown et al., 1999a, 1999b; Berry and Christopher, 2001; Christopher et al., 2003).

Support for Hypothesis (2)

Importantly, no direct causal relationship has been established that shows that high rates of clinical cases of UR TD in natural populations leads to higher mortality rates than in populations without apparent cases of UR TD. One reason that *M. agassizii* has been treated as an inherently pathogenic organism and measurements of seroprevalence have been relied upon so heavily in disease assessment of natural populations, may be that Koch's postulates mostly were fulfilled in two experimental inoculation studies (Brown et al., 1994, 1999) (Table 1). These studies showed that *M. agassizii* strains PS6 and 723 were causative agents of UR TD in the desert tortoise (Brown et al., 1994) and gopher tortoise (Brown et al., 1999a), respectively, and caused seroconversion in infected individuals. However, the fulfillment of Koch's postulates neither determines the extent of morbidity that a disease has on a typical individual, nor does it indicate the biological or ecological significance of a disease.

Despite the reliance on serology in the study of URTD in natural populations, much remains unknown about the progression from colonization through infection to symptomatic disease for various strains of *M. agassizii*, and particularly about the length of the tortoise antibody response (i.e. the persistence of antibody post-infection). Indeed, no study has ever shown a consistent, unerring correspondence between results from ELISA tests and diagnoses of URTD based upon clinical signs of disease, culture of nasal lavage samples, PCR of nasal lavage samples, and/or histopathology in experimental (see Table 1) (Schumacher et al., 1993; Brown et al., 1994), pet (Johnson et al., 2006), or wild desert tortoises (Jacobson et al., 1991, 1995; Lederle et al., 1997; Schumacher et al., 1997; Brown et al., 1999b; Christopher et al., 2003; Dickinson et al., 2005; Berry et al., 2006). Without specific knowledge of the progression of disease post-exposure, it is impossible to determine whether lack of consistent patterns in the data are due to time lags between the presence of *Mycoplasma* spp and the antibody response in the tortoise, or that they are due to extrinsic factors that may interact with *Mycoplasma* spp and/or the tortoise immune system to cause (or not cause) symptomatic URTD.

Drought and the nutritional status of tortoises are tightly linked, and these factors are thought to contribute to vulnerability to contract URTD (Jacobson et al., 1991; Jacobson, 1994; Lederle et al., 1997; Schumacher et al., 1997; Brown et al., 1999a, 1999b, 2002; Christopher et al., 2003). Both reduced winter and summer precipitation has been shown to have a negative impact on forage available to tortoises as well as on water availability and the associated ability of tortoises to consume dry vegetation efficiently and to discard nitrogenous waste products in urine (Nagy and Medica, 1986; Peterson, 1996; Longshore et al., 2003).

Most reports of high rates of clinical URTD, including observations in the western Mojave in 1989, have been correlated with concurrent or immediately preceding years of low rainfall or “short-term” drought conditions (e.g. Berry, 1990; Peterson, 1994; Christopher et al., 2003; Hereford et al., 2006). Reports of very low rates of clinical cases of URTD appear to correlate with periods of average or above-average rainfall and/or with geographic locations that receive relatively high average annual rainfall (e.g. populations in the Eastern/Northeastern Mojave seem to have lower rates of clinical cases of URTD in comparison to those in the Western Mojave) (e.g. Lederle et al., 1997; Dickenson et al., 2005). Mechanisms through which environmental factors such as rainfall, temperatures, seasonality, and vegetation type may affect the prevalence of URTD in tortoise populations are unknown. For example increased prevalence of *Mycoplasma* spp in host populations and/or interaction between the presence of *Mycoplasma* spp and host immunocompetence may influence the manifestation of clinical disease.

In gopher tortoise populations, correlations between exposure to *Mycoplasma* spp and apparent population declines appears to be variable among geographic locations and often transient, when viewed over a time frame of roughly 10 years (McCoy et al., 2007). Current data suggests a similar pattern of variability in the population-level presence and affects of *Mycoplasma* spp in desert tortoise populations. Past studies have documented geographic, and possibly temporal, differences in the relationship between seroprevalence and prevalence of individuals with symptomatic URTD in desert tortoise populations (Lederle et al., 1997; Schumacher et al., 1997; Brown et al., 1999b; Christopher et al., 2003; Dickenson et al., 2005; Berry et al., 2006). However, sampling

methods have been shown to bias estimates of population exposure to *Mycoplasma* spp in gopher tortoise populations (McCoy et al., 2007). For Mojave desert tortoise populations, it is similarly difficult to draw general conclusions, in this case, about disease prevalence, from disparate studies each focusing on only one or a few localities within the Mojave desert.

There are also frequent, but untested, references in the literature that non-mycoplasmal pathogens may interact with *M. agassizii* to cause URTD (desert tortoise: Jacobson et al., 1991, 1995; Jacobson 1994; Brown et al., 1994, 2002; Snipes et al., 1995; Christopher et al., 2003; Johnson et al., 2006; gopher tortoise: Brown et al., 1999a; McLaughlin et al., 2000). For example, several studies have found that clinical symptoms of URTD appear to correlate with the presence and/or the amount of the bacterium species, *Pasteurella testudinis*, isolated from the respiratory tract (Snipes et al., 1995; Jacobson et al., 1991; McLaughlin et al., 2000; Dickinson et al., 2001; Christopher et al., 2003). Multiple species of gram-negative bacteria also have been isolated more frequently from gopher tortoises with URTD than tortoises without URTD (McLaughlin et al., 2000).

Strains of *M. agassizii* also appear to differ in pathogenicity, or their ability to cause disease in the gopher tortoise (Brown et al., 1999a, 2002). Despite the recognition of diversity in strains of *M. agassizii*, only two strains of *M. agassizii* (PS6 and 723), and one strain of *M. testudineum* (BH29T) have been shown to be pathogenic in the desert and/or gopher tortoise (Brown et al., 1994, 1999a, 2002, 2004). Experimental infection with strains known to be pathogenic does not cause consistent morbidity and/or mortality in all animals (Brown et al., 1999a, 2002; Rostal, unpublished data; Hunter et al., 2008).

Potential evolution of virulence in Mycoplasma spp

Mycoplasma infections in other vertebrate hosts are known to include strains of varying virulence (e.g. Stipkovits and Kempf, 1996). Antigenic variation, including variation in the expression of adhesion proteins, in mycoplasmas can involve both reversible and non-reversible genetic changes that effect recognition by the host immune system and virulence (Razin et al., 1998; Rosengarten et al., 2000). Inherent differences in, and/or evolution of, virulence in species and strains of *Mycoplasma* may be important for finding a pattern to the spatial and temporal heterogeneity of mycoplasmosis in Mojave desert tortoises. In particular, different conditions of host densities, and of factors that could influence average immune resistance in tortoise populations (e.g. forage and water availability), may act as different selection pressures on the evolution of virulence in *Mycoplasma* spp (sensu Ewald, 1994; Nesse and Williams, 1994). In addition, the presence of competing strains of *Mycoplasma* spp within single tortoise hosts could also affect the evolution of *Mycoplasma* spp virulence (sensu Read and Taylor, 2001).

“Natural” antibodies as an innate immune parameter

Natural antibodies in the desert tortoise

Hunter et al. (2008) tested blood serum samples by polyclonal ELISA from 17 captive, egg-reared desert tortoises that had never been exposed to *Mycoplasma* spp, and they found varying levels of *M. agassizii*-specific antibody. The antibody levels of these known “negative” tortoises were sometimes as high as antibody levels of tortoises that were diagnosed as “positive” by the monoclonal ELISA developed by Schumacher et al. (1993) and by Western blot (Hunter et al., 2008). Furthermore, Hunter et al. (2008) showed that Western blots of surmised “positive” and “negative” animals produce

antibodies with distinct antigen binding patterns. Negative tortoises may have relatively high ELISA titers (previously described as “background” antibody levels), but produce only a relatively small number of different types of antibodies specific to certain *M. agassizii* proteins, or antigens (Hunter et al., 2008; Schumacher et al., 1993). In contrast, naturally or experimentally infected tortoises mounting a true induced antibody response to *M. agassizii*, produce antibodies specific to a much larger array of *M. agassizii* proteins, or antigens (Hunter et al., 2008; Schumacher et al., 1993). Because of considerable overlap in ELISA titers of mycoplasmosis-positive and mycoplasmosis-negative animals, a Western blot may be used as a confirmatory test to an ELISA, to measure whether a tortoise is mounting a true induced immune response to *Mycoplasma* spp (Hunter et al., 2008).

M. agassizii-specific antibodies from the 17 uninfected tortoises were of the IgM isotype (Hunter et al., 2008). which is consistent with what is known of natural antibodies in humans, mice, and other vertebrate species (Avrameas, 1991; Casali and Schettino, 1996; Boes, 2000; Baumgarth et al., 2005). Natural antibodies found in other ectotherms have also been exclusively of the IgM isotype (Gonzalez et al., 1988; Marchalonis et al., 1993; Flajnik and Rumfelt, 2000; Morrison et al., 2005; Madsen et al., 2007). While induced antibodies are made in an adaptive immune response to natural or experimental infection, “natural” antibodies are produced by a separate lineage of B-cells (termed B-1 cells in mammals) and are not induced by infection (Avrameas, 1991; Casali and Schettino, 1996; Boes, 2000; Baumgarth et al., 2005). Because they are constantly present in the blood serum, they are often considered to be part of the innate (not adaptive) immune system (Avrameas, 1991; Casali and Schettino, 1996; Boes, 2000;

Ochsenbein and Zinkernagel, 2000; Baumgarth et al., 2005). Unlike induced antibodies, natural antibodies are encoded by germ line gene segments (Baccala et al., 1989; Casali and Notkins, 1989; Kantor and Herzenberg, 1993; Boes, 2000; Baumgarth et al., 2005), and genetic differences among individuals appear to influence the amount of these antibodies produced (Hardy and Hayakawa, 1994; Ochsenbein and Zinkernagel, 2000; Sinyakov et al., 2002; Paramentier et al., 2004; Kachamakova et al., 2006). Natural antibodies have a restricted repertoire compared to the diversity of induced antibodies, but natural antibodies tend to be polyreactive and often bind to molecules conserved among classes of common pathogens (Gonzalez et al., 1988; Baccala et al., 1989; Casali and Notkins, 1989; Boes, 2000; Flajnik and Rumfelt, 2000; Baumgarth et al., 2005).

Although several possible functions of natural antibodies have been hypothesized within the literature (e.g. Avrameas, 1991; Flajnik and Rumfelt, 2000), natural antibodies have been shown to be protective, or involved in protective immunity, in a wide range of vertebrate species, including humans (Ben-Aissa-Fennira et al., 1998; suggested in Kohler et al., 2003), mice (Briles et al., 1981; Szu et al., 1983; Ochsenbein et al., 1999; Baumgarth et al., 2000, 2005), bony fish (Sinyakov et al., 2002; Magnadóttir, 2006), and sharks (suggested in Marchalonis et al., 1993; Flajnik and Rumfelt, 2000). While natural antibodies tend to be of lower affinity and more polyreactive (Boes, 2000; Baumgarth et al., 2005), they also have been shown to augment adaptive immune responses (Ehrenstein et al., 1998; Boes, 2000; Ochsenbein and Zinkernagel, 2000), and are considered to be an important link between innate and adaptive immune responses (Ochsenbein and Zinkernagel, 2000).

”Background” levels of *M. agassizii*-specific antibodies, interpreted as natural antibody levels by Hunter et al. (2008), have been described in previous studies (Brown et al., 1994; Schumacher et al., 1993). Specifically, Schumacher et al.’s (1993) Western blot data showed that both a negative control tortoise and a pre-inoculation tortoise (subsequently infected with *M. agassizii*), produced antibodies that bound to *M. agassizii* proteins (Hunter et al., 2008). Furthermore, Schumacher et al. (1993) found that negative animals had relatively high “background” antibody levels to other species of *Mycoplasma*, especially to *M. testudinis* and *M. gallisepticum*. Surprisingly, the background antibody levels to these two species of *Mycoplasma* were equal to, or higher than, antibody levels to *M. agassizii*. This demonstration of relatively high levels of antibodies that react with multiple pathogens is consistent with the general polyreactivity of natural antibodies (Guilbert et al., 1982; Gonzalez et al., 1988; Marchalonis et al., 1993; Boes, 2000; Flajnik and Rumfelt, 2000; Paramentier et al., 2004; Baumgarth et al., 2005). Desert tortoise natural antibodies may bind to antigens conserved across species of *Mycoplasma*.

Green sea turtles (*Chelonia mydas*) also appear to have high levels of “background” IgM antibodies that bind to pathogens and/or protein antigens. One of two individuals of *C. mydas* immunized with a common experimental antigen, 2,4-dinitrophenylated bovine serum albumin (DNP-BSA), had relatively high titers of IgM (possibly natural IgM), but not IgY, prior to immunization (Herbst and Klein, 1995). Natural antibody levels to DNP in *C. mydas* had also been noted in an earlier study of turtle antibodies (Benedict and Pollard, 1972). In still another experiment, individuals of *C. mydas* with FPHV-specific antibodies (fibropapillomatosis-associated herpes virus)

did not test ELISA positive for IgY specific for another virus, LETV (lung-eye-trachea disease-associated herpes virus) (Coberly et al., 2001). However, the same turtles tested positive for antibody (including IgM) that reacted with LETV-infected cultured cells via an immunohistochemistry assay (Coberly et al., 2001). This observation suggests the possible presence of polyreactive IgM (either natural or induced) that binds multiple types of virus.

Necessity to reinterpret past studies of mycoplasmosis in the desert tortoise

Because of the uncertainty in the interpretation of chelonian antibody levels as indicators of current (or recent past) infection, research on mycoplasmosis in the desert tortoise warrants reinterpretation. Past infection studies have selected tortoises with the lowest “background” levels of antibodies as the “best” negative control specimens (Schumacher et al., 1993; Brown et al., 1994), which may have introduced bias into the research design. Therefore, no distinction has been made between the ability of *M. agassizii* to cause disease in all experimental desert tortoises versus in tortoises with low relative levels of natural antibodies. Tortoises with low levels of natural antibodies may have low innate resistance towards *M. agassizii*.

Consistent with the hypothesis that low levels of natural antibody to *M. agassizii* may increase a tortoise’s susceptibility to infection, Brown et al. (1994) found that two of their tortoises, experimentally infected with exudates from clinically ill individuals, had relatively high pre-inoculation levels of antibody and failed to show an induced antibody response. This result suggests that tortoises with relatively low innate levels of *M. agassizii*-specific antibody mount relatively large induced antibody responses to experimental infection with *M. agassizii*. In domestic chickens, a positive correlation has

been found between high levels of natural antibody and the ability to produce high levels of specific antibody following experimental immunization (Paramentier et al., 2004).

Comparative importance of innate immune mechanisms across vertebrate taxa

Previously overlooked, high levels of tortoise natural antibodies specific to *M. agassizii* may be viewed as an example of immunologists' general preoccupation with the mammalian adaptive immune system and a failure to consider innate immune mechanisms as equally important defense strategies (Turner, 1994b; Janeway et al., 2005). Different components of adaptive and innate immunity may have varying importance among taxa, and the relative importance of these immune mechanisms may vary with such traits as metabolic strategies (e.g., homeothermy vs. ectothermy) or life-history traits (e.g. Turner, 1994a; Hsu et al., 1998; Norris and Evans, 2000; Bayne and Gerwick, 2001; Hangalapura et al., 2003). In contrast to mammals, ectotherms may rely more heavily on innate immune mechanisms (ectotherms: Avtalion et al., 1976; fish: Manning, 1994; Bayne and Gerwick, 2001; Magnadóttir, 2006; amphibians: Horton, 1994; reptiles: Jurd, 1994). In particular, high levels of natural antibodies have been documented in sharks (up to 40 or 50% of total blood serum protein) (Marchalonis et al., 1993; Flajnik and Rumfelt, 2000), bony fish (Gonzalez et al., 1988; Morrison et al., 2005), and one reptile (the water python *Liasis fuscus*) (Madsen et al., 2007). In fish, high "background" antibody levels to a wide variety of natural pathogens, but not to artificial protein antigens, have long been recognized as a relatively common phenomenon (Avtalion et al., 1976; Sinyakov et al., 2002).

Lack of knowledge of mechanisms of antibody production in the desert tortoise

The potential biological significance of “background” levels of *M. agassizii*-specific antibody in desert tortoise blood sera emphasizes the need to investigate the full diversity of possible innate and adaptive immune mechanisms tortoises employ to combat common pathogens. As a first step, additional research characterizing the relative production and possible protective function of both induced and natural antibodies against *Mycoplasma* spp in the desert tortoise is necessary.

Mammalian B-1 cells, which produce natural antibodies, do not appear to undergo affinity maturation, exhibit a memory response, or consistently exhibit isotype switching according to the same mechanisms used by “conventional” B-2 cells, which produce induced antibodies (Hardy and Hayakawa, 1994; Baumgarth et al., 2005). Separate B cell lineages have not been discovered and/or adequately described in the desert tortoise or any other chelonian (but see Andreas and Ambrosius, 1989).

The number and relative proportions of possible multiple light chains (Pilström et al., 1998) in the induced and natural antibody repertoire of the desert tortoise needs to be investigated and understood (but see Schumacher and Klein, unpublished data, cited in Schumacher et al., 1993). However, the monoclonal antibody (“HL673”) to one tortoise light chain used in the current ELISA (Schumacher et al., 1993) has been used widely in basic chelonian immunology and disease research as well as in assessments of desert tortoise “disease status” (Schumacher et al., 1993; Brown et al., 1994, 2004; Herbst and Klein, 1995; Coberly et al., 2001). If chelonians generally, or some individual chelonian species, produce more than one type of light chain, then ELISA results using “HL673” may have been erroneously interpreted as reflecting total antibody production in these studies of various chelonian species.

Assessing the individual-, and population- level variation in natural antibody production, using a combination of Western blotting and ELISA techniques, will help clarify the ecological and evolutionary importance of *Mycoplasma* spp in natural tortoise populations. If different populations have different patterns of natural antibody production, this could indicate different evolutionary relationships with species or strains of *Mycoplasma* across the range of the desert tortoise. Such patterns need to be analyzed in relation to different environmental conditions and/or the presence of other pathogens, both of which may interact with *Mycoplasma* spp to exacerbate the ecological importance of disease in certain tortoise populations. In addition, the degree of protection conferred by both natural and induced antibodies needs elucidation to substantiate hypotheses concerning the dynamics of the host-immune response, as well as hypotheses about the prevalence of mycoplasmosis and disease.

Euthanasia as a disease management practice

Euthanasia policy of displaced seropositive tortoises

Since approximately the time of the official listing of the Mojave population of the desert tortoises, the practice of euthanasia of displaced, “diseased” animals has been supported in both peer-reviewed and non-peer-reviewed publications. In a 1989 conference on URTD, cited in the Recovery Plan (FWS, 1994), the idea of removing sick tortoises from natural populations was first discussed (Berry and Slone, 1989). As the monoclonal ELISA test (Schumacher et al., 1993) became essentially the only measure of diagnosing URTD, seropositive animals were viewed as potential carriers of disease regardless of whether they exhibited symptoms of URTD. The treatment of large numbers of seropositive, displaced tortoises became a complicated management problem.

Jacobson et al. (1995) wrote “*The Desert Tortoise (Mojave Population) Recovery Plan . . . fails to recommend what to do with ill or subclinically affected tortoises. Because there is no known drug therapy for long-term improvement of tortoises with URTD, euthanasia rather than relocation should be considered for such tortoises. Healthy appearing, sero-positive tortoises from populations in which URTD has been seen should be considered infectious, and should not be released into areas where URTD has not been observed.*”

Managers took that advice and euthanasia became common in some areas of the Mojave. (Tracy, unpublished data).

Culling/euthanasia in disease management

Recent reviews of disease management programs include a focused discussion of the theoretical underpinnings of culling programs and their relationship to current epidemiological models (e.g. Schaubert and Woolf, 2003; Lloyd-Smith et al., 2005; Sterner and Smith, 2006). Epidemiological models of URTD in the desert tortoise are completely absent in the literature. Currently, the population- and individual-level data necessary for the formulation of epidemiological models does not exist. Necessary data should include the progression, possible recovery, and possible resistance of individuals as well as the prevalence and spread of *Mycoplasma* spp within and among desert tortoise populations. Additionally, culling has been used for economic reasons in domestic animals, but it has never been used in humans, and it should be thoroughly considered before use in rare or endangered species.

Epidemiological modeling of human, livestock, and wildlife diseases has been based on what are now known as “SIR” (“susceptible-infected-recovered”) models of the host population (Anderson and May, 1979, 1991; Heesterbeck and Roberts, 1995;

Swinton et al., 2002). Transmission of bacterial or viral pathogens can either be modeled as density-dependent transmission or as frequency-dependent transmission (reviewed in Swinton et al., 2002; Lloyd-Smith et al., 2005). Density-dependent disease models use transmission rates that vary deterministically with host population size. Culling, sterilization, and temporary birth control have been used in attempts to control density-dependent diseases by reducing both infected (I) and susceptible (S) subpopulations of the host population (Smith and Cheeseman, 2002; Lloyd-Smith et al., 2005; Swinton et al., 2002; Wobeser, 2002). Frequency-dependent disease models use constant transmission rates. Diseases consistent with this model may be managed by reducing only the number of infected (I) animals, which has been less frequently employed in wildlife management (sensu Lloyd-Smith et al., 2005).

None of these culling strategies considers the possible context-dependency (e.g. the influence of environmental variables) in the manifestation of disease (sensu Wobeser, 2002, 2006). To manage a disease associated with context-dependent opportunistic pathogens, the management of environmental parameters and not (solely) the size and density of the host population may be the best strategy to reduce the incidence of disease.

Cost and benefits unique to sensitive species

The ethical and social concerns associated with euthanasia of domestic and wild animal species are intrinsically hard to quantify, because objections to culling are largely based on economics, and individual values pertaining to animal welfare, the value of wildlife, and biodiversity (McCallum and Hocking, 2005; Kitching et al., 2006). Explicit cost-benefit analyses are often used to justify culling programs of agricultural and wildlife species (McCallum and Hocking, 2005; Hasonova and Pavlik, 2006). The

“costs” of culling should logically be less than the “costs” of the disease in any particular population. Cost and benefit considerations of disease management via culling in species of conservation concern require a longer time scale than the time scale used to calculate immediate economic returns of an agricultural animal population. The cost of reducing biodiversity due to the culling is considered to be much greater for threatened species than for agricultural species, but there are no established ways to “calculate” the worth, and the loss, of this type of biodiversity (Sterner and Smith, 2006). Brown et al. (2002) emphasized the trade-off between the potential spread of disease, and the loss in reproduction and genetic diversity in the desert tortoise. We propose that, in a threatened species such as the desert tortoise, increases in mortality and decreases in potential reproduction due to culling logically should be less than the population-wide increase in mortality and/or decrease in reproduction and recruitment associated with a disease. Importantly, no such data exist for the population-level effect of mycoplasmosis or URTD in the desert tortoise.

Problems with euthanasia/culling specific to URTD in desert tortoises

The euthanasia policy in desert tortoises assumed that managers could accurately distinguish between infected and susceptible individuals. This assumption is largely undermined by the recognition of high natural antibody levels in desert tortoises coupled with the current reliance on an ELISA to diagnose tortoise health status. Subclinical *M. agassizii* infections in tortoises may be common (Brown et al., 2002). Subclinical mycoplasmal infections in various vertebrate species, including the desert tortoise, may be difficult to detect, even when using a combination of sensitive diagnostic techniques,

such as culture, PCR, and/or ELISA (Rottem, 2003; Baseman et al., 2004; Waites and Talkington, 2004).

Similarly, inaccurate diagnoses have precluded the accurate measure of transmission rates. Epidemiological models can only be applied to pathogens with measurable transmission rates. In addition, such models assume that transmission is a rate-limiting parameter in the manifestation of disease in the host population. Recent reviews suggest many animal mycoplasmas may be commensals that become opportunistic pathogens (Cassell et al., 1985; Razin et al., 1998; Simecka et al., 1992; Blanchard and Bebear, 2002; Brown, 2002; Frey, 2002; Rottem, 2003). Depending on the biology of the opportunistic pathogen, the manifestation of clinical symptoms is often more heavily influenced by environmental conditions, host physiology, and the presence of other pathogens than simply transmission of the pathogen (Cassell et al., 1985; Simecka et al., 1992; Stipkovits and Kempf, 1996; Razin et al., 1998; Waites and Talkington, 2004).

Euthanasia of tortoises testing positive for antibodies specific to *Mycoplasma* assumes that the tortoises' antibodies cannot protect them from URTD. If *Mycoplasma* spp are relatively prevalent in desert tortoise populations, then tortoises with high levels of natural and/or induced antibodies may be the individuals most resistant to the development of severe URTD. In this case, a euthanasia policy actually selects for the individuals most susceptible to URTD in the managed population.

A euthanasia policy also assumes that the native tortoise populations at translocation sites are either naïve to mycoplasmosis, or have a significantly lower proportion of infected animals than the population of translocated animals. However,

desert tortoises with URTD have been observed at the current Clark Co. translocation site (pers. observation), and no data have ever been published on either serology or on other measures of mycoplasmosis in the native tortoise population at the translocation site.

Regarding the possibility of future translocations of desert tortoises at various locations in the Mojave desert, some evidence exists of study areas with few URTD symptomatic (e.g. Lederle, 1997; Dickinson et al., 2005) and/or ELISA-positive tortoises (e.g. Berry et al., 2006). There are no published accounts of an entire, discrete, Mojave desert tortoise population that appears to be naïve to mycoplasmosis. Therefore, our current knowledge of mycoplasmosis in natural desert tortoise populations should not necessarily preclude the translocation of ELISA-positive animals into certain populations. However, the possible introduction of different strains and/or species of *Mycoplasma* with intentional or accidental translocations is a serious concern that should become a focus of scientific research and possibly, management policy (see Table 3). While establishing “disease free” herds or breeding stocks is sometimes stated as a management objective in poultry and livestock (Stipkovits and Kempf, 1996; Collins and Socket, 1993), the feasibility and desirability of this objective in wild populations is questionable, and currently this management strategy does not seem to be amenable to the desert tortoise-*Mycoplasma* spp system (sensu Read and Taylor, 2001; Wobeser, 2006). There is a current dearth of data and analyses concerning the individual- and population- level deleterious effects of *M. agassizii* infection as well as the prevalence of *M. agassizii*, which is exacerbated by concerns regarding the ethics and potential loss of biodiversity due to culling a threatened species. A policy to euthanize displaced desert tortoises testing ELISA-positive to *M. agassizii* cannot be justified.

To optimally manage both displaced and resident tortoise populations, information on the health status of resident and displaced tortoise populations should ideally include information beyond prevalence of mycoplasmosis, to include a range of other potentially important pathogens and possibly other indices of stress, body condition, reproduction, and estimates of longevity.

Research Recommendations

Research of URTD in the desert tortoise has pointed out a lack of adequate knowledge about the mechanisms of the tortoise immune system. The relative importance of both innate and adaptive mechanisms, in response to various infectious diseases, remains a crucial but missing link in chelonian disease research. Recent immunological research in other ectotherms, most notably in fish and amphibians, has demonstrated the existence of protective mechanisms that are of less importance, or entirely absent, in mammalian systems (Simmaco et al., 1998; Bayne and Gerwick, 2001; Plouffe et al., 2005; Rollins-Smith and Conlon, 2005; Magnadóttir, 2006).

Future research on URTD in desert tortoise needs to focus both on the interaction between the tortoise immune response and *Mycoplasma* spp at the level of the individual tortoise, and on the epidemiology of mycoplasmosis in tortoise populations across space and time (Table 3). Both these avenues of research need to address questions regarding characteristics of the *Mycoplasma* spp (e.g. variations in strain virulence) and characteristics of the tortoise immune response to *Mycoplasma* spp. The research questions outlined in Table 3 are not comprehensive, but are ones which are of immediate importance to forming new management policies that may better conserve current and future generations of the Mojave desert tortoise.

Summary

Current evidence does not support: (1) the hypothesis that levels of morbidity and/or mortality in natural populations of the desert tortoise can be predicted from measuring the prevalence of tortoises testing positive for *Mycoplasma* spp-specific antibodies, (2) the assertion that the current monoclonal ELISA test should be used as the predominant diagnostic measure of a tortoise's exposure to *Mycoplasma* spp, and (3) the euthanasia of ELISA-seropositive, displaced tortoises to limit the potential spread of *Mycoplasma* spp into natural populations. Due to our incomplete understanding of the dynamics of ectothermic immune systems in general, and of chelonian immune systems in particular, we propose more caution regarding measures of the desert tortoise immune system as proxies for tortoise health status. The desert tortoise-*Mycoplasma* spp system is a case study advocating the need to consider critically the immunological and ecological complexities of host-pathogen systems in the management of wildlife disease.

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TABLE 1. SUMMARY OF NASAL-INOCULATION EXPERIMENTS SHOWING THAT TWO ISOLATES OF *M. AGASSIZII* MOSTLY FULFILL KOCH'S POSTULATES (ADAPTED FROM BROWN ET AL. (1994) AND BROWN ET AL. (1999A)). DESERT TORTOISES WERE MONITORED 1, 3, AND 6 MONTHS POST-INOCULATION (BROWN ET AL., 1994). GOPHER TORTOISES WERE MONITORED 0, 4, 6, 8, 9, AND 12 WEEKS POST-INOCULATION (BROWN ET AL., 1999A).

Host species	<i>M. agassizii</i> isolate	Sample size	Diagnosis of control group	Diagnosis of experimental group	Reference(s)
Desert tortoise	PS6 ^a	control (n=7)			Brown et al., 1994
		^c	2/7 clinical sign	8/9 clinical sign	
		experimental (n=9)	1/7 seroconversion	9/9 seroconversion	
			0/7 culture positive	5/7 culture positive	
Gopher tortoise	723 ^b	control (n=10)			Brown et al., 1999a
		^c	0/10 clinical sign	8/9 clinical sign ^d	
		experimental (n=9)	0/10 seroconversion	9/9 seroconversion ^e	
			0/10 culture positive	8/9 culture positive ^f	
			0/10 PCR positive	3/9 ^g - 6/9 ^h PCR positive	

a. Strain PS6 was isolated from clinically ill desert tortoises.

b. Strain 723 was isolated from one clinically ill gopher tortoise.

c. Procedural controls received inoculation of culture media.

d. clinical sign at weeks post-inoculation

e. seroconversion at 12 weeks post-inoculation

f. culture at 16 weeks post-inoculation

g. PCR at 8 weeks post-inoculation

h. PCR at 12 weeks post-inoculation

TABLE 2. DATA FROM A TECHNICAL REPORT TO THE US BUREAU OF LAND MANAGEMENT (BERRY 1990), DESCRIBING DECLINES IN TORTOISE DENSITY AND INCIDENCE OF URTD ON SMALL STUDY PLOTS (1-3 mi²) IN THE WESTERN MOJAVE OF CALIFORNIA. ALTHOUGH NEVER PUBLISHED IN THE PEER-REVIEWED LITERATURE, THIS REPORT ASSERTS AN ASSOCIATION BETWEEN INCIDENCES OF URTD AND POPULATION DECLINES OF DESERT TORTOISE. THE REPORT HAS BEEN REFERRED TO WIDELY IN THE PUBLISHED LITERATURE, AND ALSO IN THE DESERT TORTOISE RECOVERY PLAN (FWS 1994). THESE OBSERVATIONS HAVE BEEN USED TO SUPPORT THE HYPOTHESIS OF A CAUSAL RELATIONSHIP BETWEEN URTD AND POPULATION DECLINES OF DESERT TORTOISE EVERYWHERE IN THE MOJAVE DESERT. ONE ADDITIONAL STUDY PLOT CITED IN BERRY (1990), THE STODDARD VALLEY PLOT, IS NOT INCLUDED HERE DUE TO INSUFFICIENT SAMPLING TO CALCULATE POPULATION DENSITY DECLINES.

Western Mojave Plots	Dates of density data	Estimated density decline	Date of URTD observations	Incidence of URTD*
Fenner Valley and Desert Tortoise Natural Area (DTNA)				
<i>Fremont Valley Plot</i>	1979-1981	63%	1989	44% (7/16)
<i>DTNA Interior Plot</i>	1979-1988	50%	1989	52% (16/31)
<i>DTNA Interpretive Center Plot</i>	1979-1989	63%	1989	10.6% (23/217)
<i>Fremont Peak Plot</i>	1977-1989	68%	1989	0-6.9% (0/29- 2/29)**
Kramer Hills Plot	1980-1987	42-59%	no formal survey	none observed
Johnson Valley Plot	1980-1986	43-67%	no formal survey	none observed
Lucerne Valley Plot	1980-1986	no statistically significant decline	1989 (incidental observations)	37.5% (3/8)

*percent symptomatic (number symptomatic/total tortoises observed)

**2 tortoises with "potential sign" of URTD = dirt caked in nares

TABLE 3. RESEARCH QUESTIONS EMPHASIZING LACK OF BASIC KNOWLEDGE OF URTD IN DESERT TORTOISE POPULATIONS, THE PERTINENT PRELIMINARY EVIDENCE FOR EACH QUESTION, AND CORRESPONDING REFERENCE(S) FOR EACH QUESTION, WHERE AVAILABLE. THIS IS NOT AN EXHAUSTIVE LIST OF UNKNOWN PATHOLOGICAL AND EPIDEMIOLOGICAL QUESTIONS AND/OR PARAMETERS, BUT IT DOES REFLECT BASIC AREAS WHERE LACK OF KNOWLEDGE OF URTD AND MYCOPLASMOSIS CREATES A VOID FOR WILDLIFE MANAGERS AND CONSERVATION BIOLOGISTS.

Topic	Question	Preliminary evidence	Reference
Pathology	Can individuals recover from mycoplasmosis and clear <i>Mycoplasma</i> spp?	"Seropositive" tortoises may convert to "seronegative" or "suspect" over time.	Lederle et al., 1997; Brown et al., 1999b
	Are some individuals genetically more resistant to mycoplasmosis and/or URTD?		
	Are tortoises with poor body condition more likely to contract mycoplasmosis and/or develop URTD?		
	Do supplements of food and water (to increase body condition), improve chances of recovery from mycoplasmosis or URTD?		
	Do some tortoises live with chronic, low levels, of <i>Mycoplasma</i> spp with little or no detrimental effects on lifespan or reproduction?	Two eastern Mojave tortoises had symptoms for only two out of five years.	Dickenson et al., 2005
	Can "silent carriers" of <i>Mycoplasma</i> spp be diagnostically distinguished from unexposed, naïve animals via culture, PCR, ELISA, or western blot?		
	Are natural and/or induced <i>Mycoplasma</i> spp - specific antibodies protective against the development of URTD?		
	Can individuals acquire complete/partial protective immunity through direct exposure and/or vaccination?		
	Are antibiotics effective in clearing symptoms and/or clearing infection?	Evidence exists of reoccurrence of clinical signs (URTD) in treated, captive tortoises.	Jacobson and McLaughlin, 1997, in Jarchow,

		2004
	Oral clarythromycin may be used as a treatment in tortoises (recommended dosage of 15 mg/kh every 2-3 days).	Wimsatt et al., 1999
	Clarithromycin is an antibiotic against <i>Mycoplasma</i> spp in humans.	Wimsatt et al., 1999
	8/10 captive tortoises remained asymptomatic for 11-78 months with a combination treatment of antibiotic and cortisone.	Jarchow, 2004
	What is the variation among <i>Mycoplasma</i> species and strains in their virulence, transmission, or other biological parameters?	
	Does the abundance of <i>Mycoplasma</i> spp (within an individual) affect the development of clinical disease?	
	Do the type, extent, and duration of the immune response to <i>Mycoplasma</i> spp affect the development of clinical disease?	
	What other immune mechanisms, in addition to natural and induced antibodies, are involved in a tortoise immune response to <i>Mycoplasma</i> spp infection?	
Epidemiology	Does the incidence of URTD and mycoplasmosis correlate with reduced recruitment rates and/or reduced survivorship?	
	What is the detection rate of tortoises that have died due to mycoplasmosis or URTD?	

Do populations vary in their susceptibility/resistance to mycoplasmosis and/or URTD?	
Does population-level variation in mycoplasmosis and/or URTD correlate with spatial and/or temporal patterns of environmental parameters (e.g. annual rainfall, annual temperatures, "short term drought")?	
Do populations show variation in natural antibody production?	<p>Inbred populations of the Galapagos hawk (<i>Buteo galapoensis</i>) had lower average levels and less variable natural antibodies than more outbred populations</p> <p style="text-align: right;">Whiteman et al., 2006</p>
Do population means of natural antibody production correlate with the respective population-wide prevalences of URTD?	

Chapter 2. Natural and induced antibodies in experimentally immunized desert tortoises (*Gopherus agassizii*): the importance of season and gender

Abstract

Captive desert tortoises were immunized with ovalbumin (OVA) in Ribi's adjuvant to induce a humoral immune response, both before and after hibernation. We observed a significant mean increase in OVA-specific antibody, and a gender-by-season interaction in the ability of desert tortoises to make an induced immune response. We observed relatively high levels of pre-existing natural antibody to OVA in all tortoises, and levels varied among individuals. There was a significant, negative relationship between an animal's natural antibody titer and the maximum increase in induced antibody titers, and a significant, positive relationship between the magnitude of long-term elevations in OVA-specific antibody titers and the maximum increase in induced titers. Both natural and long-term elevations in induced antibody titers may be important elements of the tortoise immune system, with possible influences on the ecology and evolution of host-pathogen interactions. Reliance upon natural antibodies and the persistence of induced antibodies may be an adaptation in reptiles to defend themselves from pathogens in spite of their slow metabolic rates.

Keywords

natural antibody, induced antibody, ectothermic, reptile

Introduction

While research on reptilian immune systems gained momentum in the 1970s and 1980s, interest in reptiles within the field of immunology subsequently waned (Origgi 2007) even though a more complete understanding of the reptilian immune system is recognized as being important to the study of comparative immunology of vertebrates (Origgi 2007). There is a dearth of knowledge of the diversity and relative importance of immunological mechanisms in reptiles (Stacy & Pessier 2007). Most immunological studies of chelonians have focused on adaptive immune processes (i.e., induced antibody responses and lymphoproliferation assays; Ambrosius 1976; Leceta & Zapata 1985; Muñoz & De La Fuente 2001, 2003; Herbst & Klein 1995; Work *et al.* 2001; Origgi 2007).

Recent studies in ecological immunology stress the importance of “innate”, or constitutive, immune parameters (e.g. Matson 2006; Salvante 2006; Martin *et al.* 2008). Constitutive immunity is a more precise term for “innate” immune mechanisms, and it refers to mechanisms that are not induced by direct pathogen exposure (Schmid-Hempel & Ebert 2003; Martin *et al.* 2008). It has been hypothesized that constitutive immune mechanisms may be more important to ectothermic, versus endothermic, vertebrates due to their slow metabolic rates of ectotherms and slow induced immune responses of relatively low magnitude (Bayne & Gerwick 2001; Chen *et al.* 2007).

Research of chelonian immune responses largely has focused on a relatively small number of species (e.g. *Chrysemys picta*, *Testudo hermanni*, *T. graeca*, *Chelonia mydas*, *Chelydra serpentina*, *Mauremys caspica*, *Gopherus agassizii*; Ambrosius 1976; Herbst &

Klein 1995; Mead *et al.* 1983; Schumacher *et al.* 1993; Muñoz & De la Fuente 2001, 2003; Origi 2007). Species studied to date have the ability to produce three isotypes of antibodies (IgM, IgY, and IgY Δ Fc) in response to infection or artificial immunization, with the production of IgY and IgY Δ Fc following the initial production of IgM (Chartrand *et al.* 1971; Benedict & Pollard 1972; Coe 1972; Ambrosius 1976; Herbst & Klein 1995). The temporal progression of an induced humoral, or antibody, response has not been quantified in most species, including tortoises in the genus *Gopherus* (but see Ambrosius 1976). However humoral responses in chelonians have been used as an indicator of disease status and exposure to certain antigens (e.g. Coberley *et al.* 2001; Herbst *et al.* 1998, 2008; Jacobson & Origi 2007; Sandmeier *et al.* 2009).

We focus on two possible constitutive immune parameters in desert tortoises (*Gopherus agassizii*), natural antibodies and long-term elevations of induced antibodies. We describe a generalized humoral response in the desert tortoise. We immunized tortoises with an intradermal injection of chicken egg protein (ovalbumin or OVA) in Ribi's adjuvant to induce OVA-specific antibody responses in a group of 16 captive tortoises, all of which had not previously been exposed to OVA. OVA is an immunogenic protein of moderate size, known to stimulate B-cells and produce strong humoral responses in vertebrates (Parslow 2001).

We address three aspects of production of OVA-specific antibodies in the desert tortoise. First, we determined whether immunization resulted in increased OVA-specific induced antibody production, and assessed influences of gender and season on antibody production. Second, we quantified "long-term" antibody titers one year after immunizations, and determined whether the magnitude of an individual's antibody

response affects the magnitude of “long-term” antibody titers. Third, we assessed whether the levels of natural OVA antibodies, prior to antigen exposure, affect the magnitude of the induced antibody response, and whether season and gender influence natural antibody titers.

Methods

Experimental design & treatment

Twenty captive adult tortoises, housed at the University of Nevada, Reno, were immunized intradermally either with OVA in Ribí’s adjuvant (n = 16), or with sterile saline solution (n = 4). Each animal received two 0.5 ml intradermal injections (one in each forelimb) of OVA (Sigma Chemical Co., St. Louis, MO) in Ribí’s adjuvant (Ribí ImmunoChem Research, Inc., Hamilton, MT) or sterile saline (Ribí’s adjuvant promotes humoral responses in experimental immunizations; Roitt & Delves 2001). Ten (eight experimental and two control) tortoises were immunized in November 2005 (called the winter-immunized group), prior to hibernation, and an equivalent group was immunized in April 2006 (called the spring-immunized group), after hibernation. Treatment and control groups had equal numbers of females and males. A blood sample was taken from each animal prior to treatment, to serve as an individual-specific control. All animals were “boosted” twice with OVA/Ribí’s adjuvant or sterile saline solution two and five months after the initial immunization.

Animal husbandry

All tortoises had been in captivity for at least 10 years prior to the start of the experiment. They were housed in an approved animal care facility at the University of Nevada, Reno, and maintained at a temperature range of 19° to 32° C in ambient day

length in Reno, Nevada. Tortoises were fed a diet of alfalfa and green vegetables several times weekly, and once weekly each individual was placed in a tub with water where they could take a drink and become well hydrated. Hibernation (at 13° C) was gradually induced in December, and animals were allowed to hibernate for 1.5 months. One adult female, in the spring-immunization group, was a replacement for a deceased animal, and this animal did not hibernate.

One spring-immunized female was removed from the experiment during the course of treatments, and did not receive a third immunization. Two additional animals were not available for blood collection one year later.

Blood sampling

Blood samples (2ml) were drawn subcarapacially (Hernandez-Divers *et al.* 2002) prior to treatment in November 2005. After treatment, 0.5-1.0 ml of blood was taken every two weeks until September 2006 (for the winter-immunized group) and until November 2006 (for the spring-immunized group). Finally, a blood sample was taken from all animals approximately one year after the last immunization treatment, in August 2007. All blood samples were centrifuged, and plasma was frozen at – 30° C.

Polyclonal ELISA (OVA)

OVA-specific antibodies present in frozen blood plasma samples were assayed using a polyclonal ELISA (Hunter *et al.* 2008) with the following modifications. Immulon IB 96-well plates (Thermo Fisher Scientific, Inc., Waltham, MA) were coated with 50 μ l per well of OVA antigen in PBS (10 μ g/ μ l). ELISAs were run at four dilutions of tortoise blood plasma (1:10, 1:100, 1:1000, 1:10,000). To calibrate ELISA plates were calibrated against a standard sample, run on each plate. The standard sample consisted of

plasma pooled from a separate group of 21 captive desert tortoises. Blood samples from this group were collected in August/September 2007.

Titer calculations

For each sample, absorbances were plotted (dilution vs. absorbance) on a \log_{10} scale, and a best-fit line was used to approximate the linear portion of each resultant curve. Because all tortoise samples had relatively large OVA-specific ELISA titers before immunization (natural OVA antibodies), we did not have a “negative” sample to use as a null, or “background”, absorbance. To calculate antibody titers, we instead chose a “cut-off” absorbance of one that intersected each best-fit line. Therefore, titers are (\log_{10}^{-1} - transformed) dilution values at which the corresponding absorbance, on the best-fit line, equals one.

We calculated averages based on the bi-weekly antibody titers in order to minimize the influence of non-directional, weekly fluctuations in antibody titer, the immunization schedule, a minimum lag time of four weeks in the tortoise humoral response, and individual variation in the gradual increase in antibody titers. Titer values, per individual animal, were averaged across consecutive blood sampling dates (spanning 4-10 weeks) as follows: “pre-bleed” titers = average of first three sampling dates (prior to an increase in antibody titers); post-1st immunization titer = average of sampling dates 4-6 (during which there was an average increase in antibody in response to the first immunization); post-2nd immunization titer = average of sampling dates 7-12 (during which there was an average increase in antibody in response to the second immunization); post-3rd immunization titer = average of dates 13-17 for winter group, =

average of dates 13-15 for spring group (during which there was an average increase in antibody in response to the third immunization) (Fig. 1, 2).

Animals produced the maximum antibody response after the third immunization, and the average of these maximum antibody titers were used in all statistical analyses. Similarly, “maximum increases in antibody titer” were obtained by calculating the ratio of the average antibody titer after the third immunization to the average of pre-immunization antibody titers.

Statistical analyses

Mann-Whitney U tests were used to compare the effect of two treatment groups. One-tailed tests were used to detect increases in antibody titers, due to immunization treatment. Two-tailed tests were used to detect differences between season and gender. Kruskal-Wallis tests were used to compare three or more groups, and Spearman Rank-Correlations were used to test for correlations between two variables. We used a significance level of $\alpha = 0.05$, and all statistical analyses were run in Aabel (Aabel 3, Gigawiz Ltd. Co.).

Results

General humoral response to OVA immunization

Before immunization tortoises had relatively high levels of natural antibodies to OVA (Fig. 1). A Western blot was used to confirm that tortoise antibodies were specifically binding OVA (data not shown). While variability among individual tortoises in natural antibody levels was large (pre-immunization titers in Fig. 1), natural antibody titers of the four control animals, on a per-animal basis, tended to be relatively constant over the course of an active season (Fig. 1c).

Maximum titers ($n = 15$) of induced antibodies in experimentally immunized animals were significantly elevated over pre-immunization natural antibody titers ($n = 16$) ($Z = -3.558$; $p < 0.001$) (Fig. 1). There was also a significant difference in the maximum increase in OVA-specific antibody titers between experimental ($n=15$) and control ($n = 4$) animals ($Z = -2.2$; $p = 0.014$) (see Fig. 2).

We found that gender (females: $n = 7$, males: $n = 8$) alone did not significantly affect the maximum increase in antibody titers ($Z = -1.157$; $p = 0.247$). Similarly, the season of the immunization treatment (winter: $n = 8$, spring: $n = 7$) alone did not affect the maximum increase in antibody titers ($Z = -0.926$; $p = 0.355$). However, while gender (females: $n = 4$, males: $n = 4$) did not affect the maximum increase in antibody titers in the winter-immunized group ($Z = -0.5774$, $p > 0.5$), in the spring-immunized group males ($n = 4$) had a significantly greater increase in antibody titers in comparison to females ($n = 3$) ($Z = -2.121$, $p = 0.034$) (Fig. 2).

Long-term antibody response

Antibody titers approximately one year post-treatment ($n = 14$) were significantly higher than pre-immunization antibody titers ($Z = -2.895$; $p = 0.002$) (Fig. 2). Maximum increases in antibody titer ($n = 14$) were positively correlated with antibody titers one year post-treatment ($r_s = 0.9076$; $t = 7.493$; $p < 0.01$) (Fig. 3), suggesting that immunization-induced antibodies in *G. agassizii* are long-lived or that antibody production is sustained.

Natural antibodies

Winter natural antibody levels ($n = 10$) were significantly lower than spring natural antibody levels ($n = 10$) ($Z = -1.890$; $p = 0.029$]. Gender (females: $n = 10$, males:

n = 10) did not explain the lower natural antibody titers in the winter ($Z = -0.302$; $p > 0.05$), and there was no significant gender by season interaction (winter-immunized animals (females: n = 5, males: n = 5): $Z = -1.149$; $p = 0.251$; spring-immunized animals (females: n = 5, males: n = 5): $Z = -0.522$; $p > 0.5$). Thus, gender generally, and gender in different seasons did not explain the lower levels of antibodies seen in winter.

Discussion

We discovered that *G. agassizii* have variable levels natural antibodies to OVA. Unlike conventional antibodies, natural antibodies are coded for in the genome (Baccala *et al.* 1989; Casali & Notkins 1989; Kantor & Herzenberg 1993; Baumgarth *et al.* 2005), are mainly of the IgM isotype (Casali & Schettino 1996; Baumgarth *et al.* 2005), and are thought to be an important component of innate immunity against infectious agents (Briles *et al.* 1981; Szu *et al.* 1983; Ochsenbein *et al.* 1999; Baumgarth *et al.* 2000, 2005). The existence of such antibodies reactive with the non-infectious OVA probably results from their inherent polyspecificity (Gonzalez *et al.* 1988; Flajnik & Rumfelt 2000; Baumgarth *et al.* 2005). Natural antibodies to a wide variety of antigens (including OVA) are present in non-mammalian vertebrates (e.g. Gonzalez *et al.* 1988; Flajnik & Rumfelt 2000; Sinyakov *et al.* 2002, 2006; Hangalapura *et al.* 2003; Paramentier *et al.* 2004; Madsen *et al.* 2007).

While immunization with OVA induced an antibody response, the maximum response (20- to 30-fold) was not as high as the maximum responses often measured in mammals (e.g. 100-fold titer increases; Leishman *et al.* 1998; Parslow 2001). Nonetheless, immunization induced a significant increase in OVA-specific antibodies, elevated above both individual pre-immunization titers, and above changes in antibody

titer of the procedural control animals (Fig. 1, 2). Additionally, we found three prominent, unexpected patterns. These included: (1) the failure of an antibody response in females that were immunized in the spring (Fig. 1b, 2b); (2) relatively high, individually-variable, levels of natural antibody to OVA, with a negative relationship between natural and induced antibody titers (Fig. 1c, 4); and (3) a long time-period across which antibody levels remained elevated (Fig. 1, 2, 3).

Lack of an antibody response in females immunized in spring

An effect of the season in which the animal is exposed to antigen has been observed in other immunization experiments in chelonians (reviewed in Ambrosius 1967; Leceta and Zapata 1986). However, those studies did not report an influence of gender on the humoral response, and the effects of season appear not to be consistent among species and among immune mechanisms. For example, *Testudo hermanni* exhibits delayed immunological reactivity to immunization in the fall, and not in the spring (Ambrosius 1967). *Mauremys caspica* has significant season-by-gender interactions in some but not all functions of spleen leukocytes, and regulation appears to be somewhat, but not solely, influenced by hormone levels (e.g., testosterone, estradiol, and corticosterone) (Muñoz & De la Fuente 2001).

In female desert tortoises, reproductive hormones (viz., estradiol, testosterone, and corticosterone) may modulate a physiological trade-off between the expenses of reproduction (egg production) and the expenses of a humoral immune response (sensu Saad *et al.* 1991; Zapata *et al.* 1992; Burnham *et al.* 2003; Origgi 2007). Estradiol has received much less attention than have testosterone and corticosteroids, but all three hormones may affect changes in the reptilian immune system (Burnham *et al.* 2003;

Origi 2007). In addition to a bimodal spike in estradiol production in early spring and fall (Lance *et al.* 2001, 2002), female desert tortoises also exhibit their highest relative levels in both testosterone and corticosterone in spring (Rostal *et al.* 1994; Lance *et al.* 2001). Additionally, the direct energetic cost of egg production could influence induction of humoral responses in female tortoises in the spring. By measuring only two immune parameters (i.e. natural and induced antibody levels), we are unable to conclude that females are “immunosuppressed”, or to quantify a possible shift in immune parameters (*sensu* Norris & Evans 2000; Adamo 2004 ; Salvante 2006).

Tortoises immunized in spring also did not have a delayed response to OVA. Upon second and a third exposure to OVA in summer and early fall, anti-OVA antibody levels did not increase, suggesting that females may have become tolerant to OVA (Fig. 2b). The mechanisms by which the responsiveness of the immune system to a T cell-dependent immunogen, like OVA, is possibly seasonally repressed in female tortoises are not known. Female tortoises may experience seasonal shifts in immune function that have long-term effects on immune responsiveness, making them more-or-less susceptible to certain classes of pathogen, with possibly important effects on the epidemiology of disease in wild populations.

Natural antibodies to OVA

Variation in natural antibody levels to OVA in desert tortoises may indicate variation in natural antibodies to various pathogens, and therefore, those variations could well be of ecological and evolutionary significance. Supporting this hypothesis, natural antibodies are known to be cross-reactive (Guilbert *et al.* 1982; Gonzalez *et al.* 1988), titers are thought to be genetically determined (Paramentier *et al.* 2004), and large

individual variation in titers have been reported in humans, mice, birds, and fish (e.g. Ben-Aissa-Fennira *et al.* 1998; Sinyakov *et al.* 2002; Paramentier *et al.* 2004; Kachamakova *et al.* 2006). Chickens with high levels of natural antibody to sheep red blood cells, usually also had high titers to a variety of other antigens (Parmentier *et al.* 2004). Natural antibodies are protective against pathogenic organisms in fish (*Carassius auratus*, Sinyakov *et al.* 2002) and mice (Briles *et al.* 1981; Cartner *et al.* 1998; Baumgarth *et al.* 2000).

It is possible that the diversity and quantity of natural antibody levels in natural host populations – and especially those of ectothermic vertebrates – may be indicators of pathogens of ecological and evolutionary importance. For example, when specific reactivity to a pathogen molecular motifs (sensu Briles *et al.* 1981; Baumgarth *et al.* 2005) is shown to exist in a host species' natural antibody repertoire, the diversity and quantity of natural antibody may have evolved as a response to past pathogen pressure (sensu Baumgarth *et al.* 2005; Madsen *et al.* 2007). However, with the exclusion of *M. agassizii* (Hunter *et al.* 2008), little is known about the reactivity of the desert tortoise natural antibody repertoire to a variety of possible pathogens.

The negative relationship between natural antibody titers and the magnitude of the induced antibody response (Fig. 4), suggests that natural antibodies may have a significant biological effect through epitope masking. Epitope masking refers to the binding of antigen by constitutive molecules, such as natural antibodies, which then reduce the amount of “free” antigen and the subsequent magnitude of the induced response (Janeway *et al.* 2005). For instance, there is a general, negative relationship between levels of natural antibody and levels of acquired antibody, produced in response

to vaccination with a particular antigen, in fish (Sinyakov *et al.* 2006; Sinyakov & Avtalion 2009). Madsen *et al.* (2007) also interpreted the failure of water pythons (*Liasis fuscus*) to make an induced antibody response to immunizations to such an effect of natural antibodies.

Although other mechanisms of the desert tortoise immune system may be enhanced in winter (Christopher *et al.* 1999), reduced levels of natural antibody in winter may alter host immunology and host-pathogen interactions in the desert tortoise. Changes in host susceptibility could also interact with seasonal changes in pathogen growth rates and pathogen transmission patterns (*sensu* Woodhams *et al.* 2008).

Long-lived antibody response

Long-term production of induced antibodies is mechanistically different from “classical” immunological memory, which relies on secondary exposure to a specific pathogen or antigen that activates a discrete group of memory B-cells (Slifka & Ahmed 1996). In mammals, immunoglobulin molecules have relative short half-lives (Janeway *et al.* 2005), so protracted elevation in antibody titers to pathogens have been attributed to several mechanisms including: (1) re-exposure to the pathogen or chronic infection, (2) persisting antigen-antibody complexes on follicular dendritic cells, (3) cross-reactivity with environmental or self antigens, (4) idiotypic networks, and (5) the activation of a unique group of B-cells (long-lived plasma cells) that continuously produce antibodies in the absence of additional stimulation (Slifka & Ahmed 1996).

It is possible that desert tortoises possess long-lived plasma cells that produce antibodies for extended periods. However, the half-lives of desert tortoise immunoglobulins have not been reported, so it is also possible that the persistence of high

anti-OVA titers may be due to long-lived antibody molecules. It could well be advantageous to ectothermic vertebrates with low metabolic rates and slow humoral immune responses to have persistent antibodies to ensure some resistance to previously encountered pathogens and to minimize the energetic cost of producing another round of antibodies. Herbst et al. (2008) observed a long-lived antibody response in two individuals of green sea turtle (*Chelonia mydas*) with elevated IgY levels for at least 20 months after experimental infection with chelonid fibropapillomatosis-associated herpes virus. In both mammals and fish, long-lived antibody-secreting cells have been observed in the bone marrow (of mammals) and the anterior kidney (of fish) and can produce antibody for months to years (Slifka et al. 1995; Slifka & Ahmed 1996; Manz et al. 1998; Kaattari et al. 2005). Kaattari et al. (2005) suggest that long-lived, antibody-secreting, plasma cells may be responsible for the arithmetic increases in antibody titer often observed upon secondary exposure to antigens in fish. Arithmetic increases in antibody titer are also commonly observed in reptiles (Ambrosius 1976; Jacobson & Origgi 2007; Origgi 2007). Long-lived B-cells in rainbow trout (*Oncorhynchus mykiss*) are activated by antigen, migrate to the anterior kidney, and produce all or most of the circulating levels of antibody present in the late humoral response (Bromage et al. 2004; Kaattari et al. 2005).

Implications

Both high natural antibody titers and long-term elevations in induced antibody titers suggest that constitutive immune parameters may be of relatively great importance to the desert tortoise. Understanding the interactions between constitutive and induced immunity, as well as the seasonal variations in both, could lead to a better understanding of the ecology of host-pathogen interactions in the desert tortoise, and in other reptilian

species as well. Furthermore, a more complete understanding of the full array of constitutive and induced parameters in the reptilian immune system would allow for increased accuracy in diagnosing levels of pathogen exposure and host resistance in natural populations. The evolutionary significance of a possible greater reliance on constitutive versus induced immune parameters in reptiles has not been adequately explored, but could provide insights into current and past pathogen pressure, life history trade-offs in reptiles, and the evolution of the vertebrate immune system.

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FIGURE LEGENDS

Fig. 1. Average OVA-specific antibody titers by immunization treatment. For all animals, the second and third immunizations followed the preliminary immunization by two and five months, respectively. A single sample was taken one year later, in August 2007, for both groups of animals. (a) Winter-immunized animals, (b) Spring-immunized animals, (c) Procedural control animals.

Fig. 2. Average increases in OVA-specific antibody titers by immunization treatment. Increases in antibody titers were calculated as the ratio of the average titer after an immunization in relation to the average pre-immunization antibody titer: (a) Winter-immunized animals, (b) Spring-immunized animals, (c) Procedural control animals.

Fig. 3. Relationships between each tortoise's maximum increase in OVA-specific antibody titers, and the long-term elevation in OVA-specific antibodies approximately one year after the last immunization treatment.

Fig. 4. Relationships between each experimental animal's OVA-specific natural antibody titer, and the maximum increase in antibody titers in response to OVA immunization.

FIGURE 1

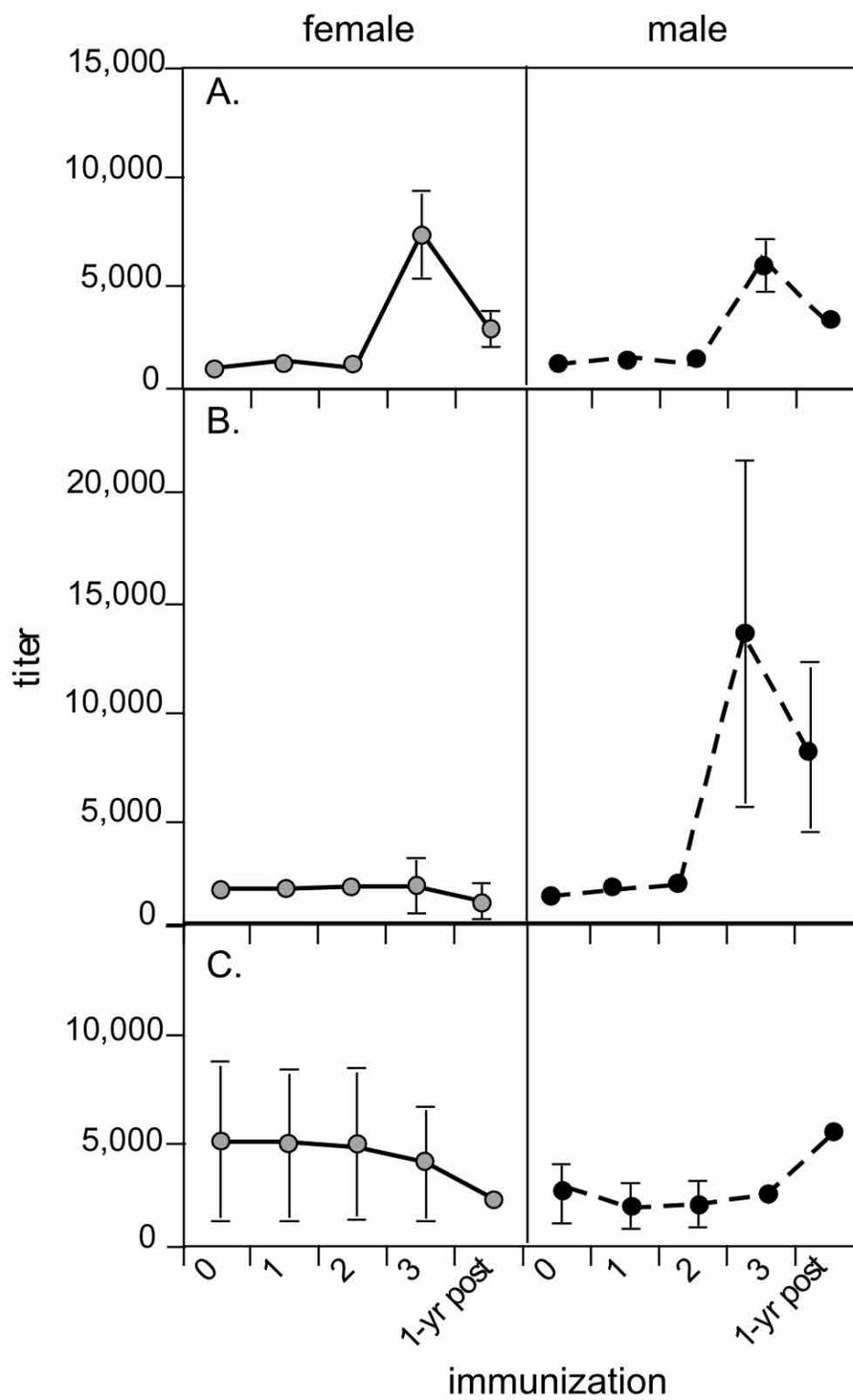


FIGURE 2

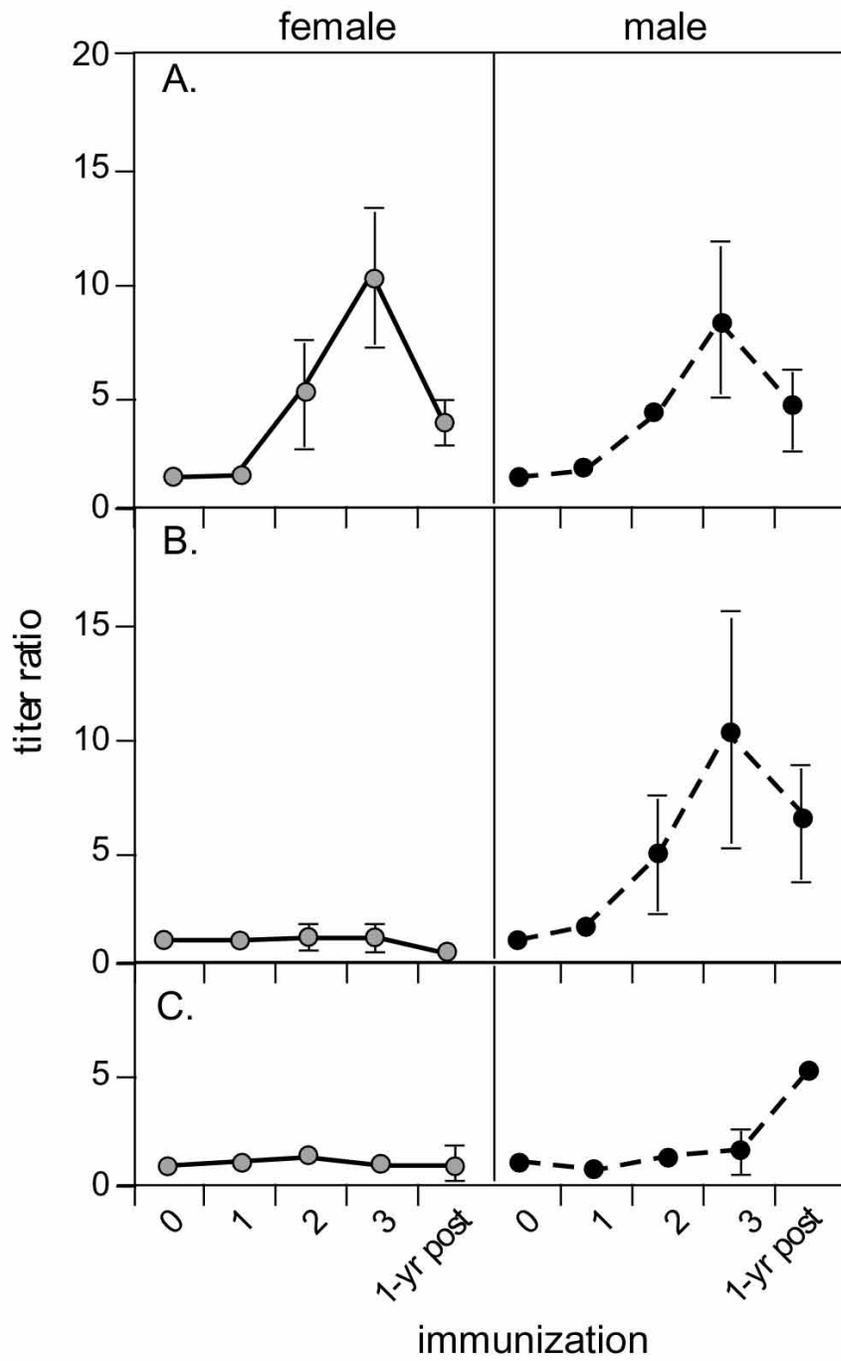


FIGURE 3

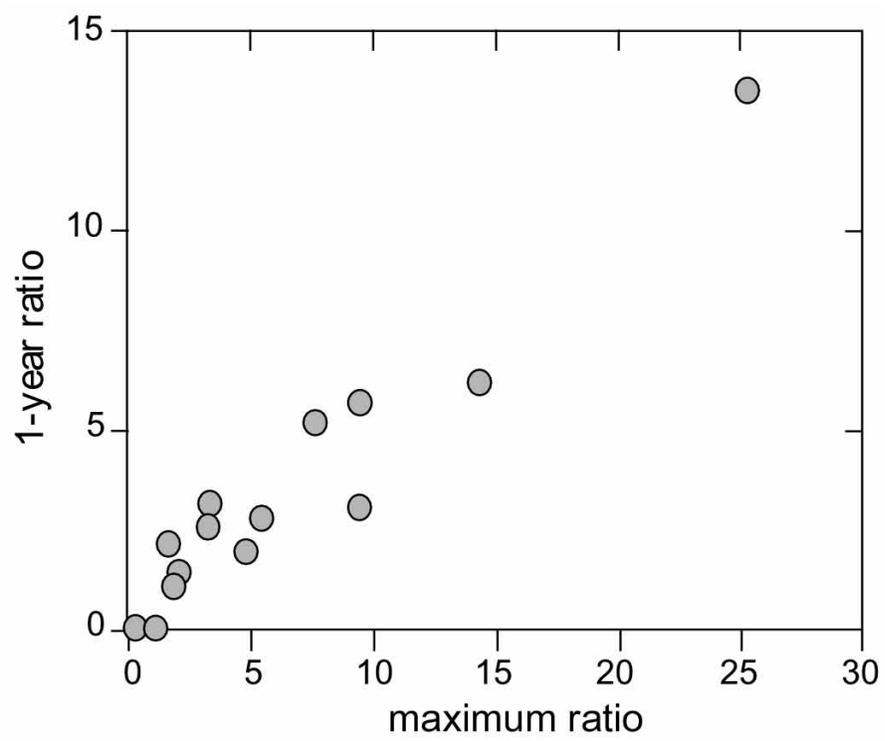
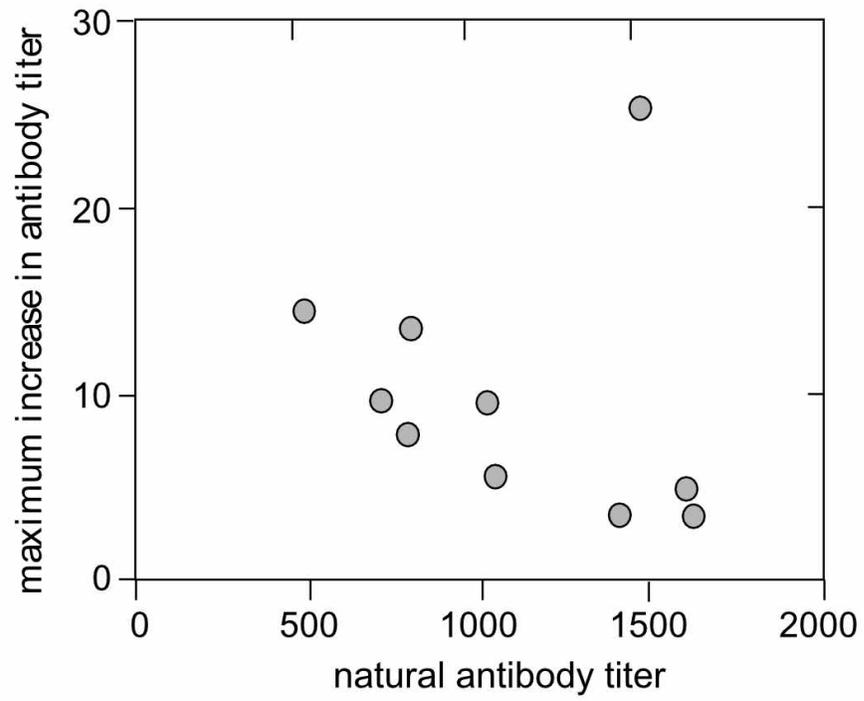


FIGURE 4



Chapter 3. Natural and acquired antibodies to *Mycoplasma agassizii* in the Mojave desert tortoise: Implications for managing a wildlife disease

Abstract

This is the first range-wide analysis of the prevalence of upper respiratory tract disease (URTD) and seroprevalence of a known, etiological agent, *Mycoplasma agassizii*, in the Mojave desert tortoise (*Gopherus agassizii*). We analyze this host-pathogen system from the viewpoint that this is a potentially complex disease, with varying dynamics over both space and time. We focus on population-level analyses (n = 24), and test for associations among prevalence of URTD, seroprevalence to *M. agassizii*, mean and standard deviations of levels of natural antibody to *M. agassizii*, genetics of tortoise populations, mean annual winter precipitation, and mean number of days below freezing. We detected significant associations between mean number of days below freezing and both prevalence of URTD and seroprevalence to *M. agassizii*. Furthermore, we detected a significant association between mean levels of natural antibody and seroprevalence to *M. agassizii*. Genetics of tortoise populations was associated with mean levels of natural antibody. We propose hypotheses, concerning possible ecological and evolutionary dynamics of the desert tortoise – *M. agassizii* system, based on these associations. We present recommendations for future research to address tests of these hypotheses.

Keywords

context-dependent, climate, wildlife disease, innate immunity, reptile

Introduction

Pathogens and parasites can pose risks to the persistence of endangered populations or species (Altizer et al. 2003, Plowright et al. 2008). However, pathogens and parasites also may be important in influencing host population dynamics, interspecific competition, levels of biodiversity, and the flow of energy in ecosystems (McCallum and Dobson 1995, Altizer et al. 2003, Hudson et al. 2006). Therefore, preserving co-evolving host-parasite populations may be a sound conservation strategy both for protecting host populations of conservation concern and conserving biodiversity in general (Altizer et al. 2003). Research aimed at understanding the impacts of anthropogenic changes – including climate change, habitat fragmentation, and expanding urban development – on these co-evolving dynamics is expected to become increasingly relevant to applied as well as basic ecological studies (*sensu* Altizer et al. 2003, Plowright et al. 2008). Unfortunately, the study of large-scale ecological factors (e.g. climatic patterns) and complex host-pathogen ecological and evolutionary dynamics are often not directly amenable to cause-and-effect experiments (Plowright et al. 2008). Plowright et al. (2008) recommend a combination of laboratory, field, and modeling techniques, a reconsideration of Hill's criteria (Hill 1965), and the consideration of multiple, competing hypotheses to investigate complex dynamics of human and wildlife diseases.

Recent epidemiological models address ways to include realistic, complex disease dynamics in natural systems. Two types of complexity in such models include the effects of population heterogeneity (in host and pathogen populations) and the relation of phenomena among different scales (reviewed in Levin et al. 1997). Therefore, new

approaches increasingly are linking within- and between-host dynamics of infectious diseases (Mideo et al. 2008), and these approaches can be used to examine effects of multiple within-host defense strategies, such as tolerance of a pathogen as well as resistance to pathogens (Schneider and Ayres 2008). For example, host-pathogen models have found that the inclusion of heterogeneity in the mechanisms of host defense can significantly change epidemiological predictions (e.g. Nath et al. 2008, Kramer-Schadt et al. 2009).

Here, we present analyses of data from the first, range-wide, serological survey of a potentially complex, infectious disease system in the Mojave desert tortoise (*Gopherus agassizii*). Upper respiratory tract disease (URTD) is thought to affect rates of morbidity and mortality in natural populations of the federally-threatened Mojave desert tortoise (FWS 1990, 1994, Sandmeier et al. 2009) (Table 1). The term “URTD” has been used to refer to visible signs of respiratory disease, regardless of etiological agent (Sandmeier et al. 2009), and we treat it as such throughout this paper. Concordant with important recommendations for such surveys (Plowright et al. 2008), our survey was based upon, and complements, past and current laboratory experiments (Schumacher et al. 1993, Brown et al. 1994, Hunter et al. 2008, Sandmeier et al. *submitted*). Mechanistic, epidemiological models of URTD in desert tortoise populations are absent from the literature. Our analyses were, therefore, designed to be concordant with some of the predictions of increasingly-realistic epidemiological models. We incorporate parameters deemed to be important in controlled experiments, namely serological measures of innate and adaptive host-defense and relatively high individual variation in these parameters (Table 1). Specifically, we measure one measure of the host innate immune system

(natural antibody levels), as well as population-level means and standard deviations (i.e. population-level heterogeneity) of these natural antibody levels. Desert tortoises exhibit high variability in natural antibody titers (Hunter et al. 2008, Sandmeier et al. 2009, Sandmeier et al. *submitted*). Natural antibodies have been shown to confer both/either tolerance and resistance to certain pathogens in a variety of vertebrate species, including humans (Ben-Aissa-Fennira et al. 1998), mice (Briles et al. 1981, Szu et al. 1983, Ochsenbein et al. 1999, Baumgarth et al. 2000, 2005), and fish (Sinyakov et al. 2002, Magnadóttir 2006).

We consider two climatic factors that have the potential to interact with infectious agents and/or the host immune system to exacerbate susceptibility to, or progression of, clinical disease. We investigate possible associations between mean measures of rainfall and thermal environment (viz., annual number of days below freezing, which correlates with duration of seasonal hibernation (sensu Nussear et al. 2007)) and the prevalence of URTD and seroprevalence of an identified etiological agent, *Mycoplasma agassizii* (strain PS6) (Brown et al. 1994, 2001, reviewed in Sandmeier et al. 2001). This survey is, in essence, a “snapshot” in time. All samples were collected over three years (2004-2006), which were roughly comparable, based upon climatic conditions and activity levels of desert tortoises (F. Sandmeier unpublished data, sensu Hereford et al. 2006, FWS 2009).

Similar to some other complex diseases of wildlife (e.g. chytridiomycosis in amphibians; Blaustein and Kiesecker 2002, Briggs et al. 2005, McCallum 2005) and to some other mycoplasma diseases of mammals (reviewed in Jones and Simecka 2003), many variables may influence rates morbidity in desert tortoises, due to infection with *M.*

agassizii (Table 1). Many of these variables in the desert tortoise – *M. agassizii* system are currently not quantified (Table 1). One caveat that is central to the interpretation of analyses in this study concerns the reliance upon serology to measure the exposure to *M. agassizii* in desert tortoises. Serology has been the most common approach to detect *M. agassizii* in wild desert tortoises (Sandmeier et al. 2009). However, seropositivity of an individual tortoise (presence of acquired antibodies to *M. agassizii*-PS6) does not necessarily indicate a current infection (Sandmeier et al. 2009, Sandmeier et al. *in press*, K. Hunter *unpublished data*). Gender and season of exposure, as well as innate levels of natural antibody, have been shown to influence the occurrence, and magnitude, of an acquired antibody response (Sandmeier et al. *in press*). In addition, desert tortoises have been shown to exhibit long-term increases in acquired antibody titers after exposure to a non-replicating, artificial antigen (ovalbumin) (Sandmeier et al. *in press*). Serology may detect past, but not necessarily current, infections, and it may fail to detect some instances of exposure to *M. agassizii*.

Both the ELISA and Western blot serological techniques used in this study are based upon the one strain of *M. agassizii* that has been shown to be pathogenic in desert tortoises and that is available through the American Type Culture Collection (Rockville, MD; ATCC 700616) (Brown et al. 1994, 2001). However, by using this one strain, it is possible that we could miss detecting significantly different strains of *M. agassizii*, or possible closely related species that may also cause URTD (*sensu* Brown *unpubl.ed data*). Avirulent strains may fail to produce URTD, and they also may fail to produce an acquired humoral response in the tortoise. In this circumstance, serology will not detect these strains. A commonly used, monoclonal ELISA to *M. agassizii* strain PS6 (described

in Schumacher et al. 1993, Wendland et al. 2007) is thought to measure antibody levels to other, known strains of *M. agassizii* (M. Brown, unpublished data). Similarly, and for brevity, we refer to levels of antibody measured via our polyclonal ELISA and Western blot, using *M. agassizii* strain PS6 as the antigen, as being specific to “*M. agassizii*.” In the future, direct, accurate detection of pathogen(s) (e.g. via nasal lavages and PCR/quantitative PCR) should be available as a preferable alternative to detect pathogens in natural, tortoise populations (pers. comm. K. Hunter, J. Simecka).

The goal of our study was to increase understanding of the factors involved in the seroprevalence of *M. agassizii*, and factors involved in the prevalence of URTD in natural populations of the desert tortoise in the Mojave Desert. Our specific aims were five-fold. We sought: (1) to examine patterns among gender, genetics of the tortoise, disease status, serological status, and natural antibody levels of individual tortoises, (2) to determine the relationship between a population’s levels of seroprevalence to *M. agassizii* and the prevalence of URTD, (3) to examine how the mean and standard deviation (measure of heterogeneity) of a population’s natural antibody levels, as well as genetic identity, mean winter rainfall, and mean annual days below freezing are associated with the seroprevalence to *M. agassizii* and the prevalence of URTD in tortoise populations, (4) to examine how genetics of the tortoise populations, mean winter rainfall, and mean annual days below freezing are associated with the mean and standard deviation of a population’s natural antibody levels, and (5) to form hypotheses pertaining to the ecology and evolution of the desert tortoise – *M. agassizii* system. We use these hypotheses to suggest fruitful avenues for future research. We propose that our approach (sensu Plowright et al. 2008) encompasses the suspected complexity of this wildlife disease and

may serve as a useful example for others involved in research of diseases with complex etiologies.

Methods

Sample collection

Tortoise blood samples were collected both by crews of workers hired by the U.S. Fish and Wildlife Service to monitor tortoises in designated critical habitat called Desert Wildlife Management Areas (DWMAs) (March-June 2004, 2005) (FWS 2009) and by our crews of two to eight people (March-October 2004-2006). Our crews walked systematically-placed transects during the active season for the desert tortoise, predominantly outside of DWMAs, in order to cover adequately the entire, occupied range of the Mojave desert tortoise (*sensu* Wiens 1989) (Hagerty and Tracy, *in press*). Our study area was divided into 24 relatively discrete “sampling groups”, based on the contiguity of habitat (Fig. 1b, Table 2). An attempt was made to sample approximately 20 tortoises in each sampling group (Table 2). Some migration is assumed to occur among these 24 valleys/inter-connected valleys. However, for brevity, we refer to these 24 sampling groups as tortoise “population” throughout.

Due to permitting restrictions, blood was collected from toenail clips in 2004, from toenail clips (NV sites) and via brachial venipuncture (CA sites) in 2005, and via subcarapacial venipuncture (Hernandez-Divers et al. 2002) in 2006. Methods of blood sampling did not appear to effect serological results, quantitatively nor qualitatively (F. Sandmeier unpublished data). All blood samples were kept on ice, centrifuged as soon as

possible later that day, and blood plasma was frozen at -30° F for long-term storage at the University of Nevada, Reno. At the time a blood sample was taken, geographic data (UTM coordinates and elevation) and tortoise-specific data, including body measurements and clinical signs of URTD (exudates in/around the nares, edema of the eyes, wheezing breath, abnormally lethargic behavior) (sensu Brown et al. 1994, Berry and Christopher 2001) were recorded.

Serology

ELISA. We followed the procedures for the polyclonal ELISA and Western blot, described in Hunter et al. (2008), with the following modifications. Plasma samples run on separate ELISA plates were calibrated to a standard, *M. agassizii*-negative plasma sample. This standard consisted of pooled, blood plasma, collected in August/September 2007 from a group of 21 captive desert tortoises that were never exposed to *M. agassizii*. These tortoises were raised from eggs at the University of Nevada, Las Vegas. They were transferred to the University of Nevada, Reno in 2004 and have remained unexposed to diseased tortoises. For each ELISA, absorbances were plotted (dilution vs. absorbance) on a \log_{10} scale, and a best-fit line was used to approximate the linear portion of each resultant curve. Due to levels of natural antibodies (Hunter et al. 2008), we did not have a “negative” sample to use as a null absorbance. To calculate antibody titers, we instead chose a “cut-off” absorbance of 0.5, a value that intersected each best-fit line. Titers are dilution values at which the corresponding absorbance, on the best-fit line, equals 0.5. Titer values were \log_{10} –transformed to approximate a normal distribution.

Western blot. The same standard sample was used to calibrate each set of Western blots. Banding patterns of antibodies from the plasma samples were compared to the natural antibody pattern of the standard, *M. agassizii*-negative sample. Completed Western blots were photographed with a digital camera, and Canvas (version X; ACD Systems of America 2005) was used to calibrate the exposure of all photographs. Photographs were then used to count bands and score the Western blot by one researcher (FS). Tortoise samples were assigned to one of five categories, according to the following criteria: 0 = same number of bands as the natural antibody pattern of the negative standard; 1 = one to three additional bands compared to the natural antibody pattern; 2 = five to seven additional bands, or more than seven, including very weak additional bands; 3 = more than 8 additional, strong bands; 4 = many additional bands that form a “smear” and are not possible to count individually. Categories 0 and 1 were considered to be “negative” for evidence of acquired antibodies to *M. agassizii* (i.e. exhibiting a natural antibody pattern.) Category 2 was considered to be “suspect”, and to indicate evidence of an early, late, or weak acquired antibody response specific to *M. agassizii*. Categories 3 and 4 were considered to be “positive”, and to indicate evidence of a strong, acquired antibody response specific to *M. agassizii*.

Statistical analyses

Individual-level analyses. Relationships among gender, genetics of tortoises, URTD, seropositivity (suspect/positive Western blot), and natural antibody levels were assessed using the following nonparametric statistical tests: Spearman’s rank correlation (URTD and seropositivity), contingency table analyses (gender and URTD; genetics of tortoises and URTD; gender and seropositivity; genetics of tortoises and seropositivity), Kruskal

Wallis tests (genetics of tortoises and natural antibody levels), and Mann-Whitney U tests (genetics of tortoises and seropositivity; genetics of tortoises and natural antibody levels). We also analyzed the distribution of natural antibody levels among genetics of tortoises, using a box-and-whisker plot (Fig. 3). We used $\alpha = 0.05$, and analyses were performed in Aable 3 (Gigawiz Ltd. Co. 2002).

Population-level analyses. Generalized linear models (multiple linear regression) were used to assess relationships among the population-level characteristics ($n = 24$). Four different measures of disease and host defense were modeled as dependent variables: (1) the proportion of the population with URTD, (2) the seroprevalence to *M. agassizii* within the population, (3) the mean level of natural antibodies, and (4) the standard deviation around mean levels of natural antibodies. Model variables used as dependent and independent variables are described below. We followed the recommendation of Zar (1950) (Freeman and Tukey 1950, cited in Zar 1999) to normalize population-proportions of animals with URTD and seropositive Western blots according to the formula:

$$p' = \frac{1}{2}[\arcsin(\sqrt{x/(n+1)}) + \arcsin(\sqrt{(x+1)/(n+1)})]$$

Significant models were determined by backward selection. In addition, all possible univariate models were also considered. We used $\alpha = 0.05$, analyses were performed in JMP version 5 (SAS Institute, Inc. 2002).

Model variables:

- 1) *Genetics of tortoises*: Each individual, and population of tortoises, was assigned to one of the three main, distinct, genetic clusters, based on microsatellite markers, occurring in the Mojave desert (“California”, “Las Vegas”, and “North Mojave”) (Fig. 1a) (Hagerty and Tracy *in press*).
- 2) *Mean amount of winter rainfall*: Mean winter rainfall (Nov. – April) was averaged over the 15 years preceding this study (1989-2003) from National Oceanographic and Atmospheric Administration (NOAA) weather stations with sufficient data (<http://cdo.ncdc.noaa.gov/cgi-bin/cdo/cdostnserach.pl>), situated across the Mojave desert. Thiessen polygons were created in ArcMap (version 9.2; ESRI 2006) to assign climatic values (winter rainfall, annual freezing days; see below) to individual tortoises and tortoise populations. Where tortoise populations fell within more than one Thiessen polygon, the respective climatic values were averaged. Climatic values were averaged over the 15 years as an approximation of the amount of winter rainfall experienced by an adult tortoise throughout its lifetime, or its recent history. Winter rainfall is known to affect the availability of spring-time forage available to desert tortoises (Hereford et al. 2006, Nussear et al. 2009). Hence, winter rainfall affects aspects of a tortoise’s nutritional status during the spring active season, and we hypothesized that nutritional status might affect a tortoise’s level of immunocompetence to infection with *M. agassizii* (Table 1).
- 3) *Mean of the annual number of days below freezing*: Similarly to mean winter rainfall, the annual number of freezing days were averaged over the same time-span (15 years), and obtained from the same weather stations. Thermal

differences among regions in the Mojave are known to affect the average length of hibernation of tortoises (Nussear et al. 2007). Both temperature and season are known to affect the reptilian immune system (Origgi 2007), and may be important in the desert tortoise-*M. agassizii* system.

- 4) *Proportion of animals with URTD*: We defined symptomatic disease as the presence of fresh or dried exudate in or around the nares, or on the face, of the tortoise. Due to the large number of observers conducting visual examinations of tortoises in the field, we deemed the presence/absence of exudate to be the most objective and accurate diagnosis of URTD. Statistical analyses, which also included other recorded signs of URTD, did not change the qualitative conclusions drawn from these analyses (F. Sandmeier unpublished data).
- 5) *Seroprevalence (proportion of the sample with a suspect/positive Western blot)*: A suspect/positive Western blot is an indication of the past production of acquired antibodies to *M. agassizii*, or a closely related species of *Mycoplasma*. This is an indication of past exposure to *M. agassizii*, however a negative Western blot does not necessarily indicate a lack of exposure to *M. agassizii* (sensu Hunter et al. 2008, Sandmeier et al. *submitted*). In addition, desert tortoises may produce “long-term” elevation of acquired antibodies (e.g. Sandmeier et al. *submitted*), positive serology does not necessarily indicate current infection.
- 6) *Mean level of natural antibody (ELISA of Western-blot-negative tortoises)*: Although natural antibodies may confer tolerance or resistance against *M. agassizii* infection (sensu Schneider and Ayres 2008, Hunter et al. 2008), the effect of natural antibodies to *M. agassizii* are currently unknown.

- 7) *Standard deviation of natural antibody level*: Heterogeneity in host populations has, theoretically, been shown to affect epidemiological patterns (e.g. Nath et al. 2008, Kramer-Schadt et al. 2009).

Results

Disease and immune status of individual tortoises

In total, plasma samples from 664 tortoises were tested by ELISA and/or Western blot. Most, but not all, samples had a sufficient volume of plasma to test by both serological techniques. Six hundred samples were tested by Western blot, and 584 samples were tested by ELISA. Complete information on signs of URTD (including an unobstructed view of the nares) was available for 648 animals. Twenty-seven out of 648 tortoises showed signs of recent discharge of exudate in or around the nares. Tortoises with URTD correlated seropositivity to *M. agassizii* among individual animals (Spearman's $r_s = 0.262$, $p < 0.001$).

URTD. Gender did not have a significant affect on an individual's expression of URTD ($\chi^2 = 1.764$, $p = 0.184$), but genetics of tortoises did have a significant effect ($\chi^2 = 8.445$, $p = 0.015$). Compared to tortoises in the Las Vegas and North Mojave genetic groups tortoises in the California genetic group were less likely to have URTD, but this relationship was not significant by nonparametric comparisons (Mann-Whitney U: compared to the Las Vegas group: $z = -0.342$, p (two-tailed) > 0.5 ; compared to the North Mojave group: $z = -1.004$, p (two-tailed) $= 0.315$).

Seropositivity. Females were more likely to be seropositive to *M. agassizii* than were males ($\chi^2 = 6.6449$, $p = 0.006$). Genetics of tortoises also had a significant effect on an individual's probability of being seropositive to *M. agassizii* ($\chi^2 = 58.013$, $p < 0.001$). Tortoises in the California genetic group had a lower probability of being seropositive than did those of the Las Vegas and the North Mojave groups (Mann-Whitney U: compared to the Las Vegas group: $z = -2.587$, p (two-tailed) = 0.01; compared to the North Mojave group: $z = -3.945$, $p < 0.001$). The Las Vegas and North Mojave cluster did not differ from each other in levels of seropositivity to *M. agassizii* (Mann-Whitney U: $z = -1.310$, $p = 0.190$).

Natural antibody levels. Females had lower natural antibody levels (Mann-Whitney U: $z = -2.437$, p (two-tailed) = 0.015). Natural antibody titers also differed by genetic cluster (Kruskal Wallis: $\chi^2 = 5.991$, $p < 0.001$). The California cluster had significantly lower natural antibody levels than the tortoises in the Las Vegas and the North Mojave cluster (Mann-Whitney U: Las Vegas: $z = -4.717$, p (two-tailed) < 0.001; North Mojave: $z = -5.503$, $p < 0.001$). The Las Vegas and North Mojave cluster did not differ from each other in natural antibody levels (Mann-Whitney U: $z = -1.222$, $p = 0.222$).

The lowest natural antibody levels of the California genetic group did not overlap with the lowest levels of the Las Vegas and North Mojave genetic groups (Fig. 3). Similarly, the highest levels of natural antibody in the Las Vegas and North Mojave genetic group did not overlap with the highest levels of the California genetic group (Fig. 3).

Disease and seroprevalence in populations (n=24)

Within each population (Table 2), the proportion of animals with URTD ranged from 0 – 22.2%. The proportion of animals seropositive to *M. agassizii* ranged from 0 – 73.3% (Fig. 2a). The proportion of animals with URTD and seroprevalence to *M. agassizii* were positively associated with each other, among populations (adjusted $R^2 = 0.588$, $p < 0.0001$) (Fig. 3). Mean levels of natural antibody titers (\log_{10} – transformed) ranged from 3.67 – 4.41 (Fig. 2b).

Generalized linear models for populations

Proportion with URTD. Mean annual days below freezing ($R^2 = 0.434$, $p < 0.0005$) was significantly associated with the proportion of the population with URTD (Table 3).

Seroprevalence to M. agassizii. Mean annual days below freezing was significantly associated with seroprevalence to *M. agassizii* ($R^2 = 0.422$, $p < 0.0006$) (Table 3). In a separate model, mean natural antibody levels were also positively associated with seroprevalence to *M. agassizii* ($R^2 = 0.232$, $p < 0.0173$) (Table 3).

Mean natural antibody titer. Genetics of tortoises ($R^2 = 0.594$, $p < 0.001$) was significantly associated with a population's mean natural antibody level (Table 3). In addition, a model including mean annual days below freezing was also significantly associated with mean natural antibody levels ($R^2 = 0.295$, $p < 0.0061$), but indicators of a lack of fit were also significant (Lack of Fit: F-ratio = 23.160, $p = 0.012$). A model including both genetics of tortoises and mean days below freezing was significant

(adjusted $R^2 = 0.593$, $p < 0.001$), but mean annual days below freezing was no longer a significant variable ($p = 0.102$).

Discussion

Individual-level analyses

Females had lower levels of natural antibody, and they had a higher probability of exhibiting an acquired antibody response to *M. agassizii*. A negative association between the level of pre-existing natural antibody and the production of acquired antibodies has been detected in desert tortoises exposed to an artificial antigen (ovalbumin) (Sandmeier et al. *submitted*). This same kind of relationship between levels of natural and acquired antibodies is possibly a common phenomenon in fish (Sinyakov et al. 2006, Sinyakov and Avtalion 2009). Because we detected no gender bias in URTD, it seems unlikely that female tortoises would have higher rates of colonization or infection with *M. agassizii*. Instead, females simply may be more likely than males to make an acquired immune response to sufficient exposure to *M. agassizii*. Diagnostic tools to detect *M. agassizii* directly in the respiratory tract are needed to test this hypothesis (Table 5). In addition, only tortoises that belong to the California genetic group showed lower levels of seroprevalence to *M. agassizii*, as well as lower levels of natural antibody. The California genetic group comprises the western- and southern-most portions of the Mojave Desert (Fig. 1a). This pattern of reduced detection of disease was unexpected due to previous reports of seemingly more-pronounced incidence of URTD in this portion of the Mojave (Sandmeier et al. 2009). However, URTD was not absent from this region, and patterns

of disease are not expected to be temporally static. Additionally, it may be that evidence of disease should be less among individuals from populations that have gone through a recent epidemic-driven reduction in population size. Population-level analyses reflect similar patterns, and hypotheses pertaining to these patterns are discussed in more detail in the following section.

Population-level analyses

We focus on population-level analyses for a number of reasons inherent both to this system (Table 1) and to the sampling protocol. Namely, signs of URTD are intermittent, acquired antibodies may remain elevated for a long periods of time (sensu Sandmeier et al. *submitted*), there may be a negative relationship between levels of natural and acquired antibodies (sensu Sandmeier et al. *submitted*), and there is considerable variability in mean levels of natural antibodies in these populations. In addition, tortoises occur in different densities across the Mojave (FWS 2009). Different movement patterns may occur in various regions, due to different topography and levels of anthropogenic land-use and development (FWS 1994). The landscape was not sampled evenly with respect to season and year, due to fiscal limitations on the number of people employed, and the time required to sample such a large number of tortoises across the Mojave Desert. Analyses of population-level proportions of URTD and seroprevalence, as well as means and variabilities of natural antibody levels, tends to aggregate and smooth much of the variance inherent in samples of individual tortoises over three years. In addition, population-level summary statistics of disease and immune status fit spatially and temporally with available weather data which also is scaled broadly in both space and time.

As expected by the positive association between the population seroprevalence to *M. agassizii* and the proportion of tortoises with URTD (Fig. 3), similar relationships with independent variables were detected for both these variables (Table 3). Mean annual days below freezing was positively associated with a population's seroprevalence to *M. agassizii* and with its proportion of animals with URTD ($R^2 \sim 0.42$ and ~ 0.43 , respectively.) Mean levels of natural antibodies were also positively associated with the seroprevalence to *M. agassizii* ($R^2 \sim 0.23$).

Mean natural antibody levels of populations were most closely associated with the genetics of the tortoise population ($R^2 \sim 0.60$). Mean natural antibody levels may also be associated with the mean annual days below freezing, but the significance of this analysis was inconclusive (Table 3). The genetic history and distribution of tortoise populations may constrain production of natural antibodies. Thus, the California genetic group historically may have lower mean levels of natural antibody than do the Las Vegas and North Mojave genetic groups (sensu Hagerty and Tracy *in press*). Taken together, these results suggest that the mean annual number of days below freezing (indicating the length of hibernation; sensu Nussear et al. 2007), may be involved in disease dynamics.

Mean winter rainfall, and the associated average hydration and nutritional status of tortoises in a given population, was relatively less important than length of hibernation as an indicator of disease and mean levels of natural antibodies. However, levels of precipitation in the Mojave vary dramatically over time (Hereford et al. 2006), and the relationships that we detected in this survey may not be temporally consistent.

Hypotheses

We use the term “virulence” to refer to the degree of morbidity, or signs of UR TD, caused by *M. agassizii*. The general fitness effects of a desert tortoise’s infection with *M. agassizii* have not been quantified (Table 1). Of the variables that we considered most likely to be associated with the incidence of disease in natural populations, only mean number of days below freezing experienced by a population were significantly associated with the prevalence of UR TD and with the seroprevalence to *M. agassizii*. In addition, mean annual days below freezing may be associated with mean natural antibody levels (although the association was inconclusive), and mean natural antibody levels were also associated with levels of seroprevalence. Numerous interpretations of these data are plausible, and here we attempt to suggest the full breadth of hypotheses that are currently consistent with our findings (sensu Plowright et al. 2008). We organize these multiple hypotheses according to assumptions that must be made in their formulation. We focus on the first hypothesis, in order to expand on the possible mechanisms underlying it. However, we recognize the importance of the assumptions underlying this first hypothesis, and relax these assumptions in the subsequent alternative hypotheses. We conclude with recommendations for future research, aimed at testing these assumptions, and, ultimately, the hypotheses themselves.

1. Immune function (mean natural antibody levels) and prevalence of *M. agassizii* are functionally related, and this current relationship has been influenced by evolution of the desert tortoise, *M. agassizii*, or both (i.e. co-evolution) (Table 4). [Assumptions: (1) UR TD and seroprevalence measure prevalence (or possibly prevalence and virulence; sensu Van Baalen 1998) of *M. agassizii*. (2) Natural antibodies provide defense against deleterious effects of exposure to *M. agassizii*.

(3) Evolutionary dynamics of the desert tortoise and/or *M. agassizii* influence the epidemiology observed in wild populations.]

2. Evolutionary dynamics have not been important in shaping the desert tortoise-*M. agassizii* system. Instead ecological interactions (e.g. host densities, tortoise movement among local populations, current climatic conditions, etc.) are the dominant force leading to the observed epidemiological/immunological pattern.
3. *M. agassizii* may not cause URTD, but is an indicator of other pathogens (which do cause URTD). Similarly, natural antibody levels may not be involved in defense against *M. agassizii*, but they may be an indicator of other (possibly more important) defense mechanisms of the immune system. Because natural antibodies are known to be cross-reactive (Gonzalez et al. 1988, Flajnik and Rumfelt 2000, Baumgarth et al. 2005), they could also be an evolutionary adaptation to other pathogens that may be involved in URTD. The mechanisms proposed in hypothesis #1 may still be involved in driving evolution of the host and/or pathogen, despite some of the assumptions being incorrect.
4. We neither actually measure the presence of *M. agassizii* in tortoises, nor a combination of the presence and relative virulence of *M. agassizii*. The serological tests employed do not detect all strains (or all virulent strains) of *M. agassizii*. Furthermore, natural antibody levels do not provide an indication of resistance or tolerance to *M. agassizii*, or to other pathogens involved in URTD. This is essentially a null hypothesis that requires testing via mechanistic experiments to verify the assumptions underlying the first hypothesis.

Expansion of Hypothesis 1. The possible mechanisms driving the associations between mean natural antibody levels, mean annual days below freezing, and prevalence of URTD/seroprevalence of *M. agassizii* are not mutually exclusive (Table 4). We briefly expand on the four major ways in which evolutionary dynamics may drive this host-pathogen system (Table 4, dynamics 1-4).

1. Extrinsic factors (e.g. thermal regime) influence the selective pressures imposed on the tortoise population, the *M. agassizii* population, or the qualitative outcomes of their ecological and evolutionary interactions (sensu Galvani 2003). While thermal regime is thought to influence tortoise activity across the Mojave Desert (FWS 2009), it has not been included explicitly in models of extant, “preferred”, tortoise habitat (Nussear et al. 2009). However, length of hibernation, or possibly a variable associated with length of hibernation, appear to affect the desert tortoise-*M. agassizii* system. While length of hibernation may affect aspects of the tortoise immune system (e.g. Origgi 2007, Sandmeier et al. *submitted*), thermal regime also may affect aspects of *M. agassizii* (e.g. growth rate, behavior affecting contact rate among tortoises, transmissibility, etc.) (sensu Woodhams et al. 2008, Cattadori et al. 2005, Grassly and Fraser 2006). Length of hibernation also may affect emergent properties of the host-pathogen system. For example, stressful hibernation affects the fitness consequences, and hence the epidemiology, of parasitic infection in a bumblebee (*Bombus terrestris*) (Brown et al. 2003).

2. *M. agassizii* may be the dominant selective pressure, sufficient to drive the evolution of higher mean levels of natural antibodies in the host populations where it is present, or where especially virulent strains are present. Pathogens and parasites are

recognized as agents of selective pressure to drive various mechanisms of defense in natural host populations (e.g. Altizer et al. 2003, Schneider and Ayres 2008). In addition, trade-offs between the costs of defense mechanisms and other life history traits (e.g. reproduction, longevity, etc.) may lead to heterogeneity among individuals in these traits of defense against pathogenic agents within a host population (Kaitala et al. 1997).

3. Defense-traits against pathogens in the desert tortoise are driving the evolution of *M. agassizii*. In particular, host defense within a population, may result in the evolution of either increasing or decreasing virulence of the pathogen, and the direction of evolution may depend on variables such as the mechanisms of host defense, heterogeneity in defense in the host population, the specificity of pathogen strains to host types, and the levels of pathogen-induced mortality in the host (Ganusov et al. 2002, Altizer et al. 2003, Galvani et al. 2003, Mackinnon et al. 2008, Alizon et al. 2009). Host heterogeneity in defense (Fig. 3) may also result in “super-spreaders” within the host population (sensu Lloyd-Smith et al. 2005). Such “super-spreaders” (e.g. tortoises with the highest levels of natural antibodies in the northeastern Mojave) may be individuals that are more tolerant of pathogenic infection (Lloyd-Smith et al. 2005). “Super-spreaders” may allow for disease persistence, and they may affect the probability and severity of epidemics (Lloyd-Smith et al. 2005). Therefore, dynamics related to defense in individual hosts can interact with between-host dynamics and fundamentally affect large-scale epidemiological patterns (Cross et al. 2007, Alizon et al. 2009).

4. Co-evolutionary dynamics may lead to iterations over time of the mechanisms described above (sensu Janzen 1980). They may also result in more complex dynamics emerging from co-evolutionary dynamics between desert tortoise and *M. agassizii*

populations. For example, multiple, evolutionarily-stable strategies have been predicted by co-evolutionary models of host-pathogen evolution (Van Baalen 1998, Koella and Restif 2001). A relatively simple model predicts two evolutionarily-stable states: hosts with little defense infected with a prevalent, relatively avirulent pathogen, and hosts with high levels of defense infected with a rare, but virulent, pathogens (Van Baalen 1998). In addition to these multiple stable-state outcomes in host-pathogen systems with co-evolutionary dynamics, cyclic and chaotic dynamics of the system have also been predicted (Kaitala et al. 1997).

Management recommendations

Without additional diagnostic tools, controlled experiments, and large-scale field (Table 5) (sensu Plowright et al. 2008), our hypotheses cannot be tested accurately. For example, an understanding of effective immune mechanisms against *M. agassizii* in the desert tortoise, mean levels of defense among local populations, and interactions of immunocompetence with external variables (e.g. climatic conditions) will allow for predictions of disease-risk in different host populations. Similarly, improved methods to detect all strains of *M. agassizii* will allow for assessment of its historic distributions and factors affecting its prevalence and persistence.

Without needed basic information, management aimed at reducing disease may actually exacerbate disease. For example, if mean levels of defense in host populations select for greater persistence and/or virulence of *M. agassizii*, the current policy of only translocating tortoises with low levels of natural antibody (Sandmeier et al. 2009) may have variable epidemiological effects. Effects may depend on the natural antibody levels of the resident population. In populations with similarly low natural antibody levels,

extant levels of *M. agassizii* may not be affected. However, adding individuals with low natural antibody levels into populations with higher levels of natural antibody could, theoretically increase the persistence and/or the virulence of *M. agassizii* (e.g. Altizer et al. 2003, Read and Mackinnon 2008, Alizon et al. 2009).

Conclusion

The complexity of this host-pathogen system (Table 2), and complex interactions suggested by this study (Table 3), necessitate further research in order to predict the effect of extant levels of *M. agassizii* in natural populations. Additional knowledge will allow managers to assess the effect of management strategies, aimed at reducing the deleterious effects of *M. agassizii* and URTD. In addition, due to this host-pathogen system's complexity - in terms of tortoise immunology, *Mycoplasma* biology, and interaction(s) with extrinsic factors - this system may provide an empirical example of little-tested hypotheses (e.g. Table 4) concerning the ecology and evolution of dynamic, context-dependent wildlife diseases.

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TABLE 1. COMPLEXITY OF DESERT TORTOISE - *M. AGASSIZII* SYSTEM AT DIFFERENT SCALES (REVIEWED IN SANDMEIER ET AL. 2009). ALSO INCLUDED ARE FACTORS THAT ARE THOUGHT TO IMPACT CLINICAL URTD, OTHER CAUSES OF MORTALITY IN THE DESERT TORTOISE, AS WELL AS FACTORS THAT COULD INFLUENCE APPARENT DECLINES IN TORTOISE POPULATIONS. REFERENCES NOT INCLUDED IN SANDMEIER ET AL. (2009) ARE IN PARENTHESES.

Scale	Characteristics
<i>Mycoplasma</i> spp (in vertebrates)	<ul style="list-style-type: none"> - high rates of antigenic variation - infections are often chronic - immune responses can limit dissemination through body (Jones and Simecka 2003) - immune responses are often inefficient at clearing <i>Mycoplasma</i> (Jones and Simecka 2003) - immune responses can lead to immunopathology (Jones and Simecka 2003) - often "opportunistic"; can cause disease in hosts with compromised immune systems and/or have other infections - multiple strains of <i>M. agassizii</i>, with varying virulence, have been identified
Desert tortoise	<ul style="list-style-type: none"> - possible individual variability in tolerance/resistance to <i>M. agassizii</i> (via natural antibodies) - individual variability in magnitude of acquired antibody responses (Sandmeier et al. in press) - immune responses fluctuate seasonally <ul style="list-style-type: none"> - seasonal fluctuations may vary by gender (Sandmeier et al. in press) - hibernation/winter season affects distribution of immune cells - nutritional/physiological state may influence immunocompetence - unquantified rates of morbidity, mortality, and/or changes in reproductive output due to <i>M. agassizii</i> - URTD is often intermittent in individual tortoises - other pathogens (e.g. herpes virus, <i>Pasteurella testudins</i>) may contribute to/cause URTD
Tortoise population	<ul style="list-style-type: none"> - possible metapopulation dynamics (and local, cyclic population fluctuations mitigated by migration among local populations) - genetic variability among Mojave populations (Hagerty and Tracy in press) - population declines are thought to have varied, across the Mojave, in recent times
Environmental conditions	<ul style="list-style-type: none"> - large temporal and spatial fluctuations in climatic conditions (e.g. rainfall, vegetation growth rates, temperature) - predator populations have varying effect on desert tortoise populations across space and time (Nussear and Esque in press) - topography, development, other anthropogenic disturbance vary across the landscape

TABLE 2. SAMPLING POPULATIONS AND ASSOCIATED ATTRIBUTES. (SEE TEXT FOR A FULL DESCRIPTION OF ATTRIBUTE VALUES.)

Sampling population *	Genetic group	Mean annual freezing days	Mean winter rainfall (mm)	Seroprevalence	n †	Proportion URTD	n	Mean NAb ‡	st. dev. NAbs	n
Cuckwala Valley (CK)	CA	19.3	269.7	0	44	0	65	3.98	0.13	41
Chemehuevi Valley (CM)	CA	2.5	310.3	0	45	0	54	3.82	0.14	35
E Providence Mtns (EP)	CA	12	498.5	0	33	0.16	36	3.79	0.17	27
Fremont-Kramer (FK)	CA	45.7	470.7	0.059	17	0.1	19	3.9	0.23	14
Ord-Rodman (OR)	CA	35	370.5	0	15	0	15	3.67	0.24	13
Pinot Mtns (PM)	CA	27	242	0	24	0	23	4.07	0.21	17
Superior-Cronese (SC)	CA	35	370.5	0.069	31	0	33	3.74	0.2	19
W Providence Mtns. (WP)	CA	24	524.7	0	13	0	12	3.8	0.24	13
Amargosa Valley (AM)	Las Vegas	66.5	376.5	0	11	0	11	4.34	0.3	8
Eldorado Valley (EL)	Las Vegas	10.7	381	0.021	46	0	45	4.02	0.13	41
Ivanpah Valley (IV)	Las Vegas	51	497	0	13	0	13	4.04	0.24	13
Pahrump Valley (PA)	Las Vegas	81.5	352	0.125	8	0.12	8	4.41	0.32	7
Piute Valley (PI)	Las Vegas	13	356.5	0.014	73	0	72	3.95	0.1	68
Shadow Valley (SH)	Las Vegas	67	410	0	15	0	16	4.02	0.27	10
South I-15 Corridor (SI)	Las Vegas	49.3	502.3	0.273	22	0	22	4.29	0.22	15
S Las Vegas Valley (SL)	Las Vegas	34.7	454.3	0.367	30	0.12	34	4.28	0.21	16
NW Las Vegas Valley (NWL)	Las Vegas	45	315.5	0.389	18	0.11	18	4.17	0.26	11
Beaver Dam Slope (BD)	N Mojave	67	424.3	0.125	8	0	9	4.04	0.32	7
Coyote Springs (CS)	N Mojave	28.7	389	0	21	0	32	4.28	0.21	17
Gold Butte (GB)	N Mojave	54	257.5	0.155	13	0	13	4.28	0.27	10
Muddy Mountains (MD)	N Mojave	18.2	373.5	0	18	0.06	16	4.09	0.22	15
Mormon Mesa (MM)	N Mojave	54	275.5	0	35	0.03	38	4.21	0.16	29
NE Las Vegas Valley (NEL)	N Mojave	18	345	0.526	19	0.22	19	4.14	0.28	9
Red Cliffs Desert Reserve (RC)	N Mojave	60	498	0.733	30	0.19	35	4.26	0.3	8

* site abbreviations correspond to those on Fig. 1b

† "n" refers to the sample size on which the previous column's statistics are based

‡ NAb = "natural antibody"

Table 3. Regression models of population-level measures of disease and natural antibody levels in tortoise populations (n = 24). Proportions were normalized (see text), and all significant models were univariate.

Response variable	Predictor Variable	R ²	p
Proportion with URTD	mean annual days below freezing	0.422	< 0.0006
Seroprevalence	mean annual days below freezing	0.434	< 0.0005
	mean natural antibody levels	0.232	< 0.0173
Mean natural antibody levels	genetic group	0.594	< 0.0001

TABLE 4. POSSIBLE HYPOTHESES (NOT MUTUALLY EXCLUSIVE) OF EVOLUTIONARY DYNAMICS IN THE DESERT TORTOISE - *M. AGASSIZII* SYSTEM (SEE TEXT FOR A FULL EXPLANATION OF ASSUMPTIONS).

Evolutionary dynamic	Possible mechanisms	References (reviews and models of similar mechanisms)
1. Extrinsic factors driving evolution of host and pathogen separately	A. Historic distributions may affect: prevalence of <i>M. agassizii</i> (or of strains) and/or diversity of natural antibody levels in tortoise populations.	
	B. Extrinsic factors (e.g. climatic variables) may affect: traits (e.g. growth rate, virulence) of <i>M. agassizii</i> and/or natural antibody levels (and trade-offs with other physiological traits) within tortoise populations.	Cattadori et al. 2005, Grassly and Fraser 2006
2. Pathogen is driving evolution of host defense	Prevalence of <i>M. agassizii</i> (or virulent strains) is a sufficient selective pressure for:	
	A. the evolution of higher mean levels of natural antibody	Altizer et al. 2003, Schneider and Ayres 2008
	B. the maintenance of heterogeneity in levels of natural antibody in tortoise populations, via (tortoise) life-history trade-offs	Kaitala et al. 1997
3. Host is driving evolution of pathogen	Higher levels of natural antibodies tortoise populations in the northeastern Mojave is a selective pressure for:	
	A. an increase in the persistence of <i>M. agassizii</i>	Altizer et al. 2003, Lloyd-Smith et al. 2005, Read and Mackinnon 2008, Kramer-Schadt et al. 2009
	B. an increase in the virulence of <i>M. agassizii</i>	Altizer et al. 2003, Galvani et al. 2003, Mackinnon et al. 2008, Read and Mackinnon 2008, Alizon et al. 2009

Table 5. Aims of basic research necessary for testing assumptions of hypotheses presented herein, and for elucidating possible evolutionary dynamics of desert tortoise-*M. agassizii* system.

Species	Development of tools	Controlled experiments	Surveys of natural populations
<i>Mycoplasma</i> spp	Create tools to detect multiple species/strains of <i>Mycoplasma</i> in tortoise respiratory tract	<p>Quantify levels at which <i>Mycoplasma</i> can be detected in the respiratory tract</p> <p>Measure growth rates of strains/spp in various conditions (e.g. various temperatures)</p> <p>Assess fitness effects (morbidity, mortality, fecundity) of strains/spp of on individual tortoises (with respect to gender, genetic population)</p> <p>Identify constraints to evolution of virulence</p> <p>Assess interactions of strains/spp with other pathogens, nutritional status of tortoise, season, temperature, physiological stress, etc.</p> <p>Quantify possible specificity of strains/spp to the genetics of tortoises</p> <p>Assess the ability to persist in other hosts spp</p> <p>Assess the possible influence of infection with <i>Mycoplasma</i> on the disease outcome of a tortoise's infection with other pathogens (sensu Sieve et al. 2009)</p>	<p>Describe spatial/temporal distribution of species/strains of <i>Mycoplasma</i> across the Mojave</p> <p>Correlate trends in tortoise population size with the distribution of URTD and strains/spp of <i>Mycoplasma</i></p>
Desert tortoise	Create assays of pertinent innate and acquired immune mechanisms	Identify of mechanisms of tolerance/resistance	Describe spatial/temporal variation in immune parameters across natural Mojave desert tortoise populations

Quantify interaction of immune mechanisms with gender, reproductive status, age, season, temperature, nutrition, etc.

Quantify interaction of immune mechanisms with different strains/spp of *Mycoplasma* to influence their virulence and/or transmissability

Quantify costs of defense mechanisms and possible trade-offs with other life-history traits

Identify features of the habitat that may affect the qualitative outcome of coevolutionary host-pathogen dynamics

FIGURE LEGENDS

Figure 1. Mojave desert, including southern California, southern Nevada, southeastern Utah, and northwestern Arizona.

a) Tortoise point locations, and three, distinct genetic groups (Hagerty and Tracy *in press*). From left to right (west to east), the genetic groups are “California”, “Las Vegas”, and “North Mojave” (*sensu* Hagerty and Tracy *in press*).

b) Twenty-four desert tortoise sampling populations (modified from Hagerty et al. *submitted*). Points designate centroids of areas in which tortoises were sampled. Abbreviations correspond to those used in Table 2.

Figure 2. Overlapping points are desert tortoise locations, and seroprevalence and mean values were assigned according to the calculated population ($n = 24$) values (see text for more detail).

a) Seroprevalence to *M. agassizii* (i.e. the proportion of the population seropositive to *M. agassizii*). Seroprevalence was transformed to approximate a normal distribution (see text for detail).

b) Mean natural antibody levels of populations.

Figure 3. Box-and-whisker diagram of natural antibody levels of individual tortoises belonging to the three main genetic groups in the Mojave desert (California, Las Vegas, and North Mojave). Natural antibody levels are \log_{10} -transformed.

Figure 4. Population-levels ($n = 24$) of seroprevalence to *M. agassizii* versus proportion with URTD. Both the population proportions of animals with a seropositive Western blot and with URTD were transformed to approximate a normal distribution (see text for more detail).

FIGURE 1A

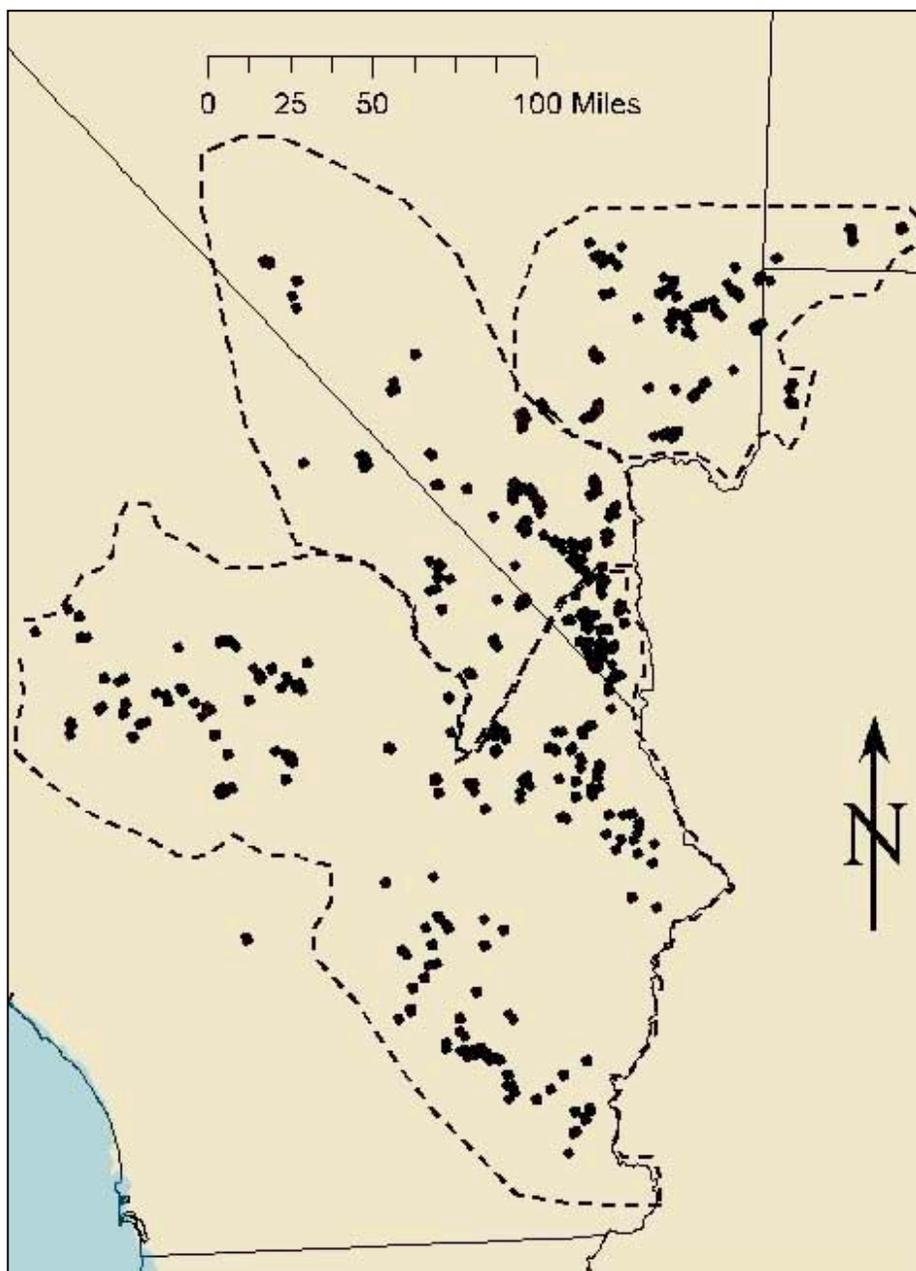


FIGURE 1B

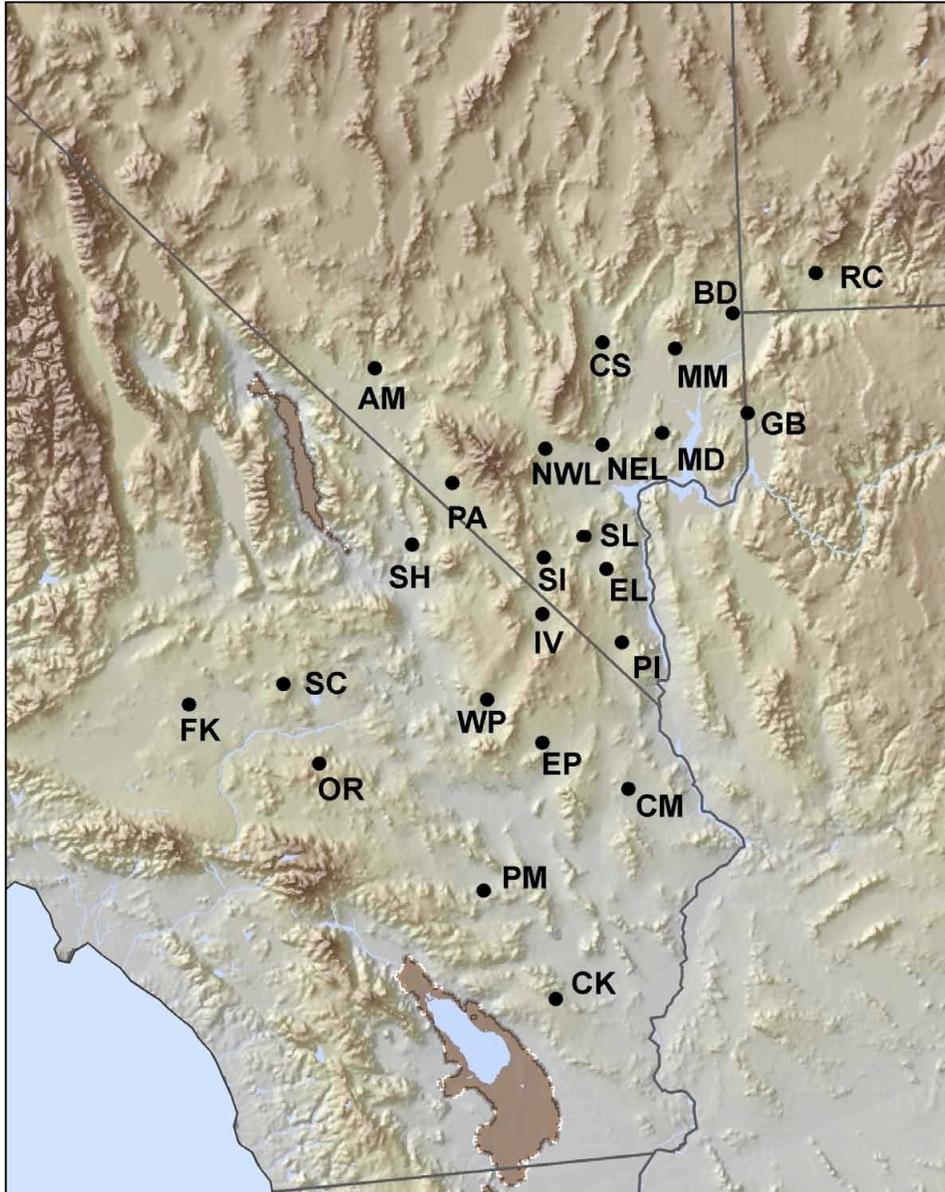


FIGURE 2A

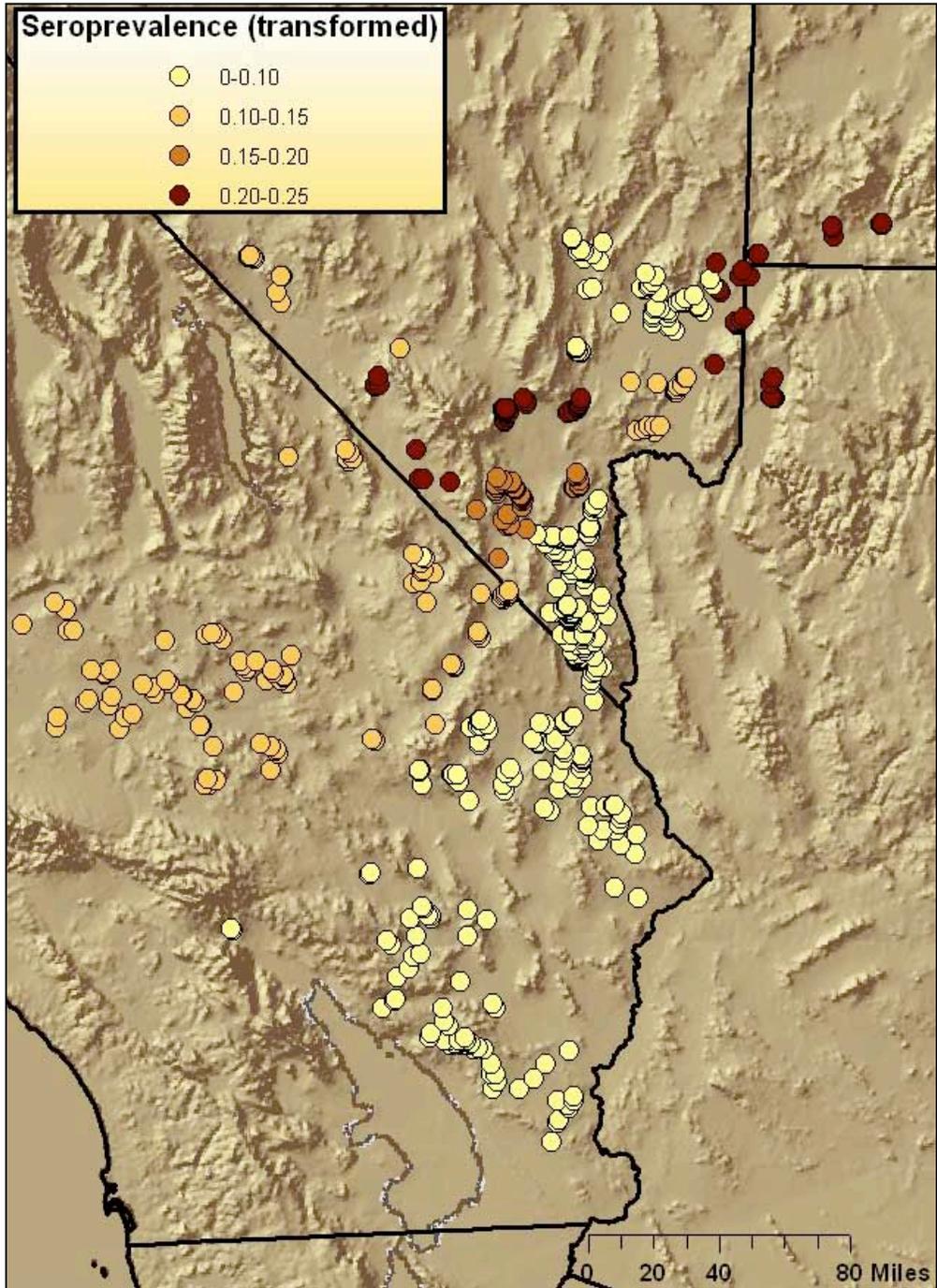


FIGURE 2B

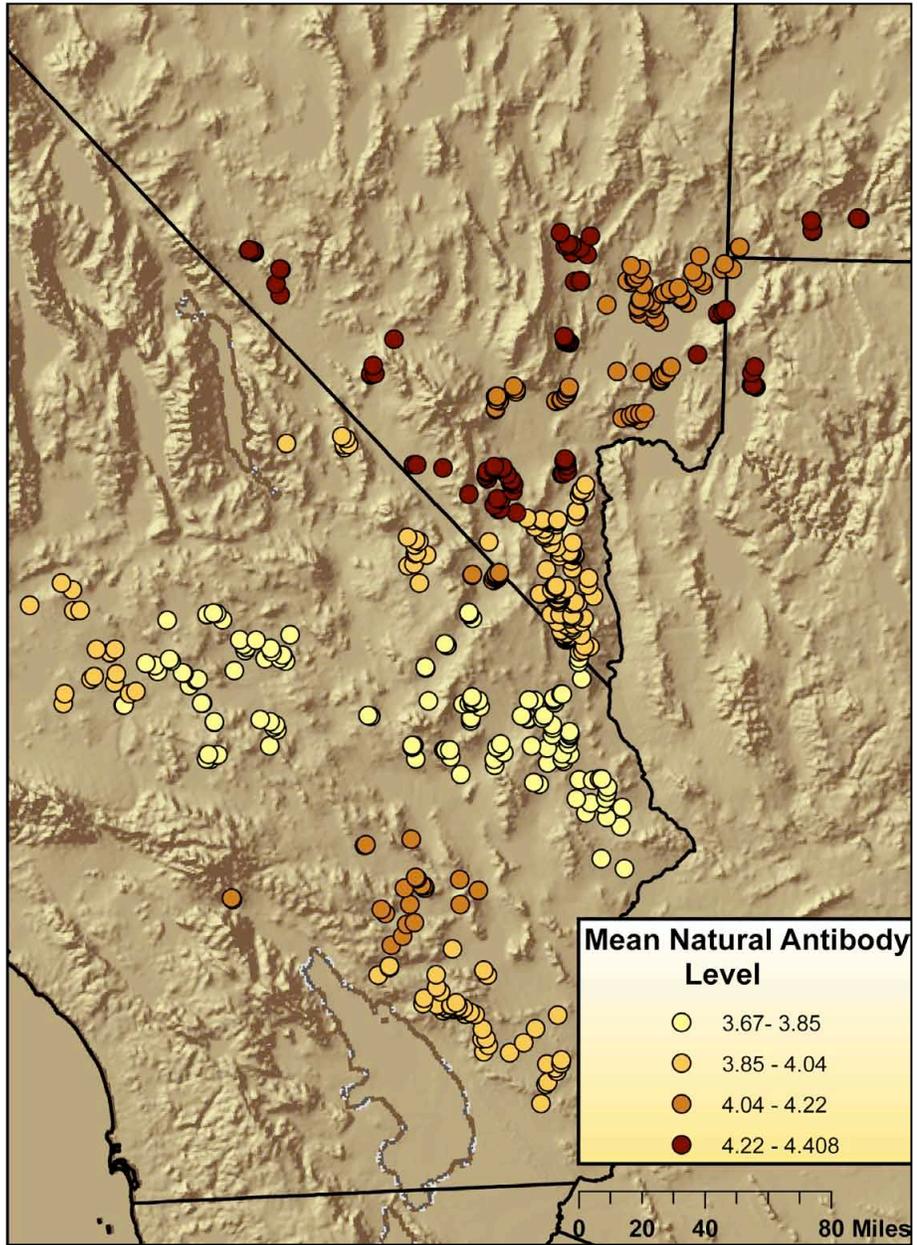


FIGURE 3

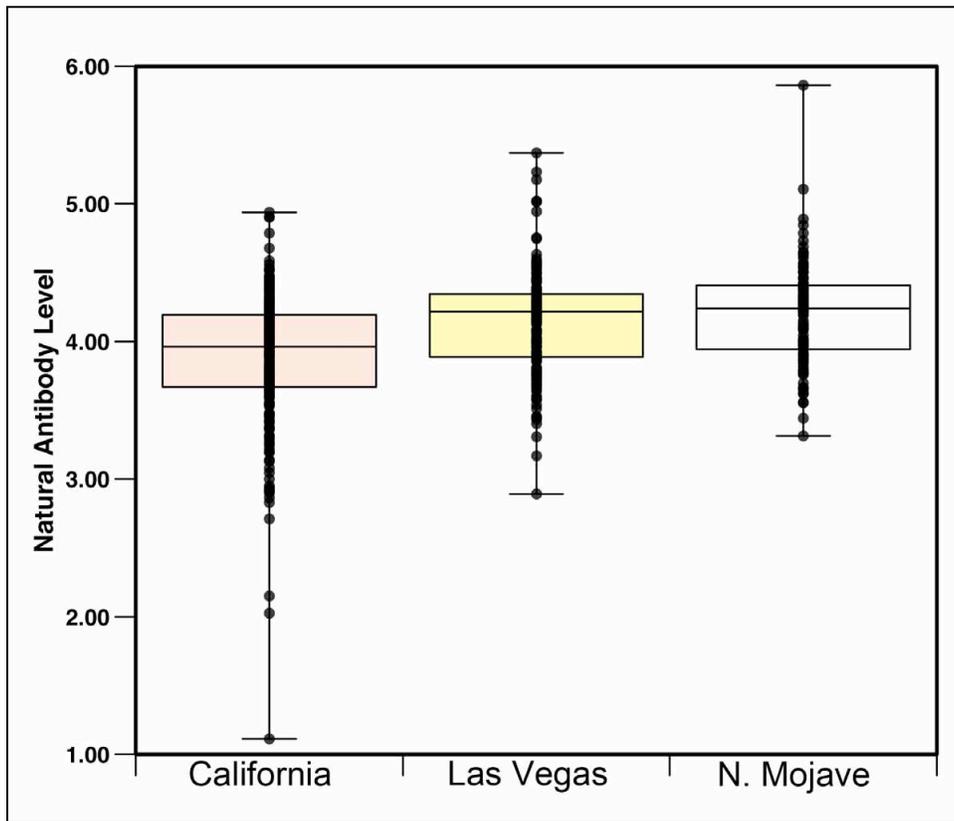


FIGURE 4

